

STUDIES OF THE CHEMILUMINESCENCE OF LUMINOL

by

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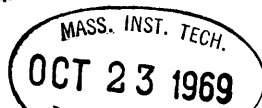
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ABSTRACT

The absorption, fluorescence, and chemiluminescence (CL) characteristics of luminol have been studied in basic dimethyl sulfoxide (DMSO) and various basic DMSO-water mixed solvents. It has been shown that the luminol dianion can be produced quantitatively in carefully deoxygenated "dry" DMSO using potassium t-butyl alcoholate (BTO) as the base. A direct correlation has been found between the intensity of CL and the concentration of luminol dianion, indicating that the dianion is the reactive species in the chemiluminescent reaction in DMSO. Increasing concentrations of water in the mixed solvents greatly reduced the CL intensity because of the decrease in luminol dianion concentration.

The absorption and fluorescence characteristics of 2,3-dihydrophthalazine-1,4-dione (PD) have been studied in water and DMSO. The 2-methyl (MePD) and 2,3-dimethyl (2,3-DiMePD) derivatives were also prepared and studied. The quantitative formation of the PD dianion was observed in deoxygenated basic DMSO. The absorption spectrum of the PD dianion is very similar to the luminol dianion absorption spectrum. The spectral characteristics of PD, MePD, and 2,3-DiMePD indicated that PD exists as the diketo-structure in water and DMSO. The absorption spectra of PD and luminol are discussed in terms of the effect of the amino group on the long wavelength transition of PD.

An attempt was made to determine the keto-enol tautomeric behavior of luminol by studying the absorption characteristics of several methyl derivatives in water and DMSO. However, any spectral shifts due to tautomeric changes which might have been occurring were obscured by solvent effects, and these results were inconclusive. Some qualitative observations are reported.

The absorption and fluorescence characteristics of the 3-aminophthalate ion (3-AP^-) in DMSO and DMSO-water mixed solvents have been studied. It is proposed that the proton-transferred excited-state of 3-AP^- is responsible for the fluorescence spectrum in DMSO. The changes which occur in the fluorescence spectrum and the fluorescence quantum yield, ϕ_f , of 3-AP^- in the mixed solvents are discussed.

The 3-AP^- fluorescence spectrum in DMSO and in each of the mixed solvents is very similar to the luminol CL spectrum in the corresponding solvent. The small differences observed for the spectra in

some of the mixed solvents were shown to be due to absorption of the CL emission by the basic luminol solution. The similarity of the 3-AP⁼ fluorescence spectrum and the CL spectrum in each of these solvents showed that both chemical and electronic excitation produce the same solvent cage for the excited-state of 3-AP⁼.

The stopped-flow technique was used to study the kinetics of the luminol CL. The results of the stopped-flow experiments in DMSO were consistent with a mechanism proposed earlier in the literature.

Thesis Supervisor: David M. Hercules

Title: Associate Professor

-5-

To Patricia

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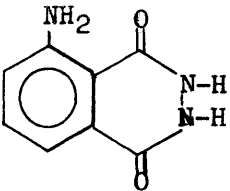
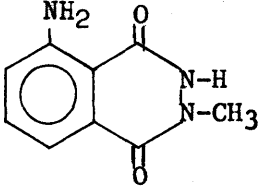
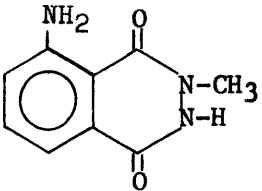
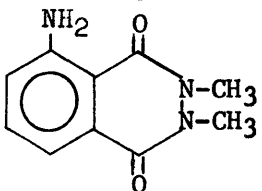
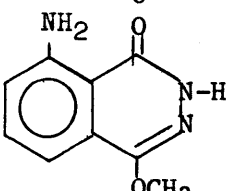
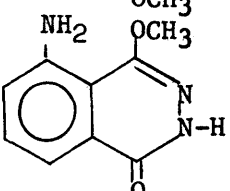
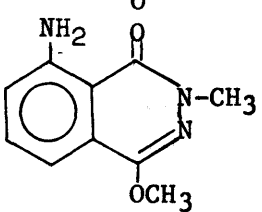
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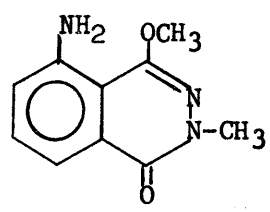
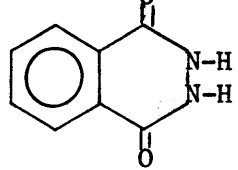
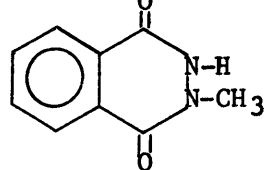
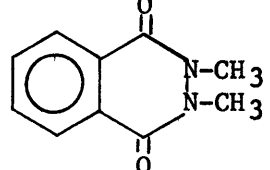
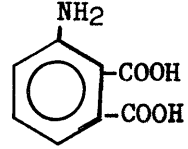
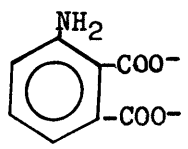
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Commonly Occurring Structures and Their Abbreviations

<u>Structure</u>	<u>Name</u>	<u>Abbreviation</u>
	5-amino-2,3-dihydrophthalazine-1,4-dione	Luminol
	5-amino-2-methylphthalazine-1,4-dione	2-MeLUM
	5-amino-3-methylphthalazine-1,4-dione	3-MeLUM
	5-amino-2,3-dimethylphthalazine-1,4-dione	2,3-DiMeLUM
	5-amino-1-methoxyphthalazine-4-one	1-MeOLUM
	5-amino-4-methoxyphthalazine-1-one	4-MeOLUM
	5-amino-1-methoxy-3-methylphthalazine-4-one	1-MeO-3-MeLUM
(CH ₃) ₂ SO	Dimethyl Sulfoxide	DMSO

Commonly Occurring Structures and Their Abbreviations

<u>Structure</u>	<u>Name</u>	<u>Abbreviation</u>
	5-amino-2-methyl-4-methoxy-phthalazine-1-one	2-Me-4-MeOLUM
	2,3-dihydrophthalazine-1,4-dione	PD
	2-methylphthalazine-1,4-dione	MePD
	2,3-dimethylphthalazine-1,4-dione	2,3-DiMePD
	3-aminophthalic acid	3-APA
	3-aminophthalate ion	3-AP ⁼
KOC(CH ₃) ₃	Potassium t-butyl Alcoholate	BTO

I. Introduction

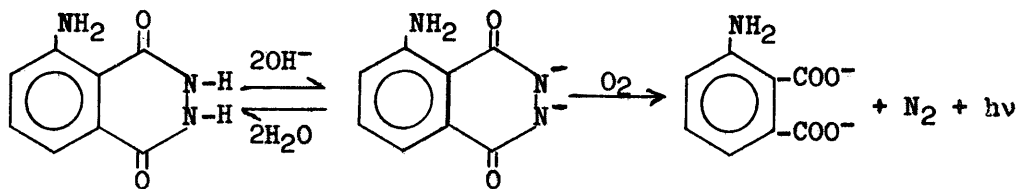
Chemiluminescence (CL) is the generation of light by chemical reactions. In these reactions a molecule capable of fluorescing is raised to an excited-state by chemical energy (1). For the reaction to be followed by the emission of visible light, the reaction must supply an energy of at least 40-80 kcal/mole. Although the CL of organic compounds in solution is a common phenomenon and many examples are found in the literature (2-7), very little is known about the chemistry involved.

The CL of luminol (5-amino-2,3-dihydrophthalazine-1,4-dione), one of the most efficient and probably the best known of the chemiluminescent compounds, was first reported in 1928 by Albrecht (8). Since this publication, there have been numerous studies concerning the nature of the luminol CL. A review of the luminol problem up to 1945 is presented by Anderson (2). The subject has been reviewed more recently by White (6), Gundermann (9), and McCapra (10).

Most of the work to date has been concerned with the chemiluminescent reaction in aqueous solution. CL ($\lambda_{\text{max}} = 425 \text{ nm}$) is observed when a basic aqueous solution of luminol is reacted with an oxidizing agent, such as potassium ferricyanide, and dissolved oxygen. Including hydrogen peroxide in the system results in an increased amount of light. However, the use of strong oxidizing agents has hampered studies of the aqueous reaction because of the extensive degradation of the reaction products and complicating side reactions.

Seliger (11) has observed that the CL yield in aqueous solution showed the same pH dependence as the fluorescence yield of 3-amino-phthalic acid. These results suggest that the 3-aminophthalate ion is the CL emitter. A detailed study of the chemiluminescent reaction of luminol with aqueous alkaline hydrogen peroxide and persulfate is reported by Rauhut and co-workers (12). Recently, Cormier and Prichard (13) investigated the luminescent peroxidation of luminol by horseradish peroxidase (HRP) using the stopped-flow technique. They report the intermediate formation of luminol radicals in the chemiluminescent reaction.

White (6) has shown that solutions of luminol in aprotic solvents, such as dimethyl sulfoxide (DMSO) and dimethyl formamide (DMF), exhibit CL ($\lambda_{\text{max}} = 490 \text{ nm}$) with only oxygen and base present. In these solvents the chemiluminescent reaction is relatively free of complications, and nitrogen and the aminophthalate ion have been identified as the products of the reaction by White, *et al.* (6, 14). They have also shown that the reaction requires two moles of base and one mole of oxygen for each mole of luminol. The general mechanism proposed by White and co-workers is presented below.



In DMSO White and Bursey (15) found a good match for the CL spectrum, the fluorescence spectrum of the total reaction product, and the fluorescence spectrum of the aminophthalate ion. Therefore, they concluded that the aminophthalate ion was the emitting species in the chemiluminescent reaction in DMSO. Analogues of luminol also produce CL spectra similar to the fluorescence spectra of the corresponding substituted carboxylic acids (16-18).

The luminol CL and the 3-aminophthalate ion fluorescence in mixtures of DMSO and water have also been studied by White and Bursey (15). They reported that in these solvent mixtures a double emission occurred in both the CL and fluorescence, and the peak positions were unchanged from the values in the pure solvents. They also noticed that with increasing amounts of water in the DMSO the water emission in the CL spectrum did not develop at the same rate as it did in the aminophthalate ion fluorescence spectrum.

One of the purposes of the present investigation was to carry out a detailed study of the spectral characteristics of luminol in basic DMSO and DMSO-water mixed solvents and determine the nature of the species responsible for the initiation of the chemiluminescent reaction. Also, a study of the spectral characteristics of the 3-aminophthalate ion in DMSO and DMSO-water mixed solvents was needed to clarify some of the results of White and Bursey (15) concerning the nature of the emitting species. Finally, it was felt that using the stopped-flow technique and monitoring the changes in the CL decay as the concentrations of the reactants were independently varied would provide some

information which would be helpful in understanding the mechanism of the reaction.

II. Experimental

A. Chemicals

All inorganic and common organic chemicals were reagent grade and were used without further purification.

Dimethyl sulfoxide (DMSO) and dimethyl formamide (DMF) were Matheson, Coleman, and Bell "Spectroquality" solvents and were used without further treatment unless otherwise noted. A sample of DMSO which had been stored over crushed KOH and vacuum distilled showed the same spectral characteristics as a sample from a freshly opened bottle. U.S. Industrial Company absolute ethanol was used without further purification.

Prepurified nitrogen and oxygen (AIRCO) were presaturated with solvent before use.

3-Aminophthalic Acid (3-APA) was precipitated from an aqueous solution of the hydrochloride (Eastman Organic Chemicals) which had been treated with decolorizing carbon, rapidly filtered and cooled to 0° C for several hours. The crystals are white, but they develop a yellow tint after about a month even when stored under vacuum in a brown bottle. It was also observed that concentrated solutions of 3-APA turn yellow after a few days and yellow crystals precipitated from aqueous solutions after about a week.

The o-aminobenzoic acid (anthranilic acid), o-dimethylamino-benzoic acid hydrochloride, m-dimethylaminobenzoic acid, p-dimethylaminobenzoic acid (Aldrich Chemical Company), m-aminobenzoic acid, and

p-aminobenzoic acid (Eastman Organic Chemicals) were used without further purification.

Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) (Eastman Organic Chemicals) was purified as the hydrobromide by several recrystallizations from concentrated hydrobromic acid (19).

The Mono-Sodium Salt of Luminol (NaLUM) was prepared according to the published procedure (20) and recrystallized three times from distilled water (21). Luminol was obtained by acidifying a concentrated solution of the sodium salt. These were stored under vacuum over P_2O_5 .

2,3-Dihydrophthalazine-1,4-dione (PD) (Aldrich Chemical Company) was dissolved in DMSO and precipitated by filtering into distilled water which was cooled to 0° C.

The Mono-Sodium Salt of PD (NaPD) was prepared, recrystallized, and stored as described above for the NaLUM. The salt crystallizes as a hydrate in long white needles. The anhydrous compound is pale yellow.

Potassium t-Butyl Alcoholate (BTO) (MSA Research Corporation) was purified before use by vacuum sublimation at 180° C and 0.01 mm.

5-Nitro-2-methylphthalazine-1,4-dione and 5-Nitro-3-methylphthalazine-1,4-dione were prepared from 3-nitrophthalic anhydride and methylhydrazine according to the procedure of Drew, Hatt, and Hobart (22). The two isomers were separated by taking advantage of the supersaturation of the 3-methyl compound in glacial acetic acid. The 2-methyl compound was recrystallized three times from acetic acid. The pale yellow crystals melted at 291° C (decomp.) (reported m.p. 292°C).

The 3-methyl compound (probably contaminated with some 2-methyl) formed pale yellow crystals, m.p. 245-255° C (decomp.) (reported m.p. 272° C), from acetic acid.

5-Amino-2-methylphthalazine-1,4-dione (2-MeLUM) and 5-Amino-3-methylphthalazine-1,4-dione (3-MeLUM) were prepared by reducing the above nitro-compounds with tin and concentrated hydrochloric acid.

The 2-methyl derivative was recrystallized twice from acetic acid and formed cream-colored crystals, m.p. 304-305° C (decomp.) (reported m.p. 308° C (22)). Two recrystallizations of the 3-methyl derivative from water resulted in pale cream-colored, long, thin needles, m.p. 296-297° C (decomp.) (reported m.p. 299° C (22)).

5-Nitro-2,3-dimethylphthalazine-1,4-dione was prepared from 3-nitrophthalic anhydride and sym-dimethylhydrazine dihydrochloride (Aldrich Chemical Company) by the method of Drew et al. (22). This compound formed pale yellow crystals, m. p. 194-195° C (reported m.p. 194-195° C), from acetic acid.

5-Amino-2,3-dimethylphthalazine-1,4-dione (2,3-DiMeLUM) was prepared by reduction of the above nitro-compound with stannous chloride and concentrated hydrochloric acid and recrystallized from water. The pale yellow crystals melted at 190-191° C (reported m.p. 192° C (22)).

2-Methylphthalazine-1,4-dione (MePD) was prepared from methyl hydrazine sulfate and phthalic anhydride according to the method of Rowe and Peters (23). The compound forms white crystals from glacial acetic acid, m.p. 237-238° C (reported m.p. 238° C (23)).

2,3-Dimethylphthalazine-1,4-dione (2,3-DiMePD) was prepared

by the method of Drew et al. (22). It was recrystallized once from water (as a hydrate) and dried in a vacuum. The long white needles of the anhydrous compound melted at 173-174° C (reported m.p. 175-176° C).

5-Nitrophthalazine-1,4-dione was prepared from 3-nitrophthalic anhydride and hydrazine hydrate according to the method of Drew and Pearman (24). This compound forms pale yellow needles, m.p. 314° C (decomp.) (reported m.p. 314° C).

It is important to note that the preparation described below for 5-nitro-1-methoxyphthalazine-4-one was run several times and always resulted in a complex mixture of products. All the nitro-compounds isolated from the mixture were obtained in small quantities. Reduction with tin and concentrated hydrochloric acid yielded even smaller amounts of the amines. The compounds were identified by the melting points reported by Drew et al. (22) (25).

5-Nitro-1-methoxyphthalazine-4-one was obtained by methylation of the silver salt of 5-nitrophthalazine-1,4-dione using methyl iodide and methyl alcohol as reported by Drew and Garwood (25). After several recrystallizations from methanol, the nitro-compound, m.p. 262-264° C (reported m.p. 269° C), was isolated. 5-Amino-1-methoxyphthalazine-4-one (1-MeOLUM) was prepared by reducing the nitro-compound as described above. The amine was recrystallized from water and melted at 233-234° C (reported m.p. 234° C).

The filtrates from the above recrystallizations of 5-nitro-1-methoxyphthalazine-4-one were evaporated to dryness and the following nitro-compounds were isolated.

1. A compound, m.p. 264-266° C, which was soluble in base. The amine was prepared as described above and recrystallized from water. It formed pale cream-colored, long, thin needles, m.p. 294-295° C (decomp.). The absorption spectrum of this compound was identical to that of 3-MeLUM described above.
2. A mixture of compounds which were insoluble in base. Several recrystallizations from methanol resulted in a nitro-compound, m.p. 168-169° C. The amine crystallized from methanol in white needles, m.p. 135-136° C. Drew and Garwood (25) reported that 5-amino-4-methoxy-2-methylphthalazine-1-one (2-Me-4-MeOLUM) melts at 136° C.
3. The filtrates from the recrystallized 5-nitro-4-methoxy-2-methylphthalazine-1-one were evaporated to dryness. The resulting substance, m.p. 100-110° C, was reduced and the amine was recrystallized from water, m.p. 220-221° C (cream-colored crystals). Pale yellow needles, m.p. 226-227° C, were obtained from methanol. Ultraviolet spectra showed the compounds were identical. This compound is believed to be 5-amino-1-methoxy-3-methylphthalazine-4-one (1-MeO-3-MeLUM), m.p. 222° C, which has also been prepared by Drew and Garwood (25).
4. A mixture of nitro-compounds which were soluble in base. Reduction of this mixture resulted in a small amount of a substance which melted at 260-262° C. This substance formed yellow crystals from ethanol, m.p. 262-264° C. The absorption

spectrum of this compound in DMSO resembled the luminol spectrum. However, the spectrum in basic DMSO was similar to the spectrum of 1-MeOLUM in basic DMSO. Since there are only four possible monomethyl derivatives with the methyl group in the hydrazide ring, and since the melting point and absorption characteristics of this compound are different from the three monomethyl derivatives described above, it is possible that this compound (m.p. 262-264° C) is the 5-amino-4-methoxyphthalazine-1-one (4-MeOLUM). In any case, this compound will be referred to as 4-MeOLUM.

B. Instrumentation

Absorption spectra were recorded on a Cary Model 14 Spectrophotometer.

A G. K. Turner Associates Model 210 "Spectro" Absolute Spectrofluorimeter was used to obtain fluorescence, excitation, and chemiluminescence spectra.

Fluorescence quantum yields were measured on the spectrofluorimeter according to the procedure of Turner (26). Quinine sulfate ($\phi_f = 0.57$ @ 25° C (26)) in 0.1 N H₂SO₄ was used as a standard. All solutions were deoxygenated by bubbling with prepurified nitrogen for 20 minutes.

C. Special Apparatus

1. Stopped-Flow System

The luminol reaction kinetics were studied using a stopped-flow system. A block diagram of the apparatus is shown in Figure II-1.

The syringe drive was a Compact Infusion Pump Model #975 (Harvard Apparatus Company, Inc.) fitted with two 100 ml Luer-Lok hypodermic syringes (Becton, Dickinson and Company). Table II-1 lists the delivery rates for several syringe drive speeds using the 100 ml syringes.

The four-jet tangential mixer is shown in Figure II-2. It was constructed from two pieces of Teflon which were bolted together and fitted with #200-1-2 Teflon Swagelok connectors (Cambridge Valve and Fitting, Inc.) as shown. The mixing jets ($3/64$ " deep x $1/16$ " wide), mixing chamber ($1/8$ " diameter) and the exit port were machined from the bottom piece. Better mixing was obtained by placing fine platinum gauze around the mixing chamber and at the exit and filling the chamber with small glass beads.

The observation tube (3.0 mm O. D. Pyrex) was masked except for a 1.0 mm slit as near to the mixing chamber as physically possible (3.0 cm.) The inside diameter of the tube was determined to be 1.73 mm. The theoretical dead time for the system was about 30 milliseconds.

The flow was stopped by rapidly closing the stopcock between the observation tube and the uptake syringe. A microswitch

Figure II-1

Block Diagram of the Stopped-Flow Apparatus

Key

V - Three-way Valve

M - Teflon Mixer and Observation Tube

PM - Photomultiplier

AMP. - Amplifier

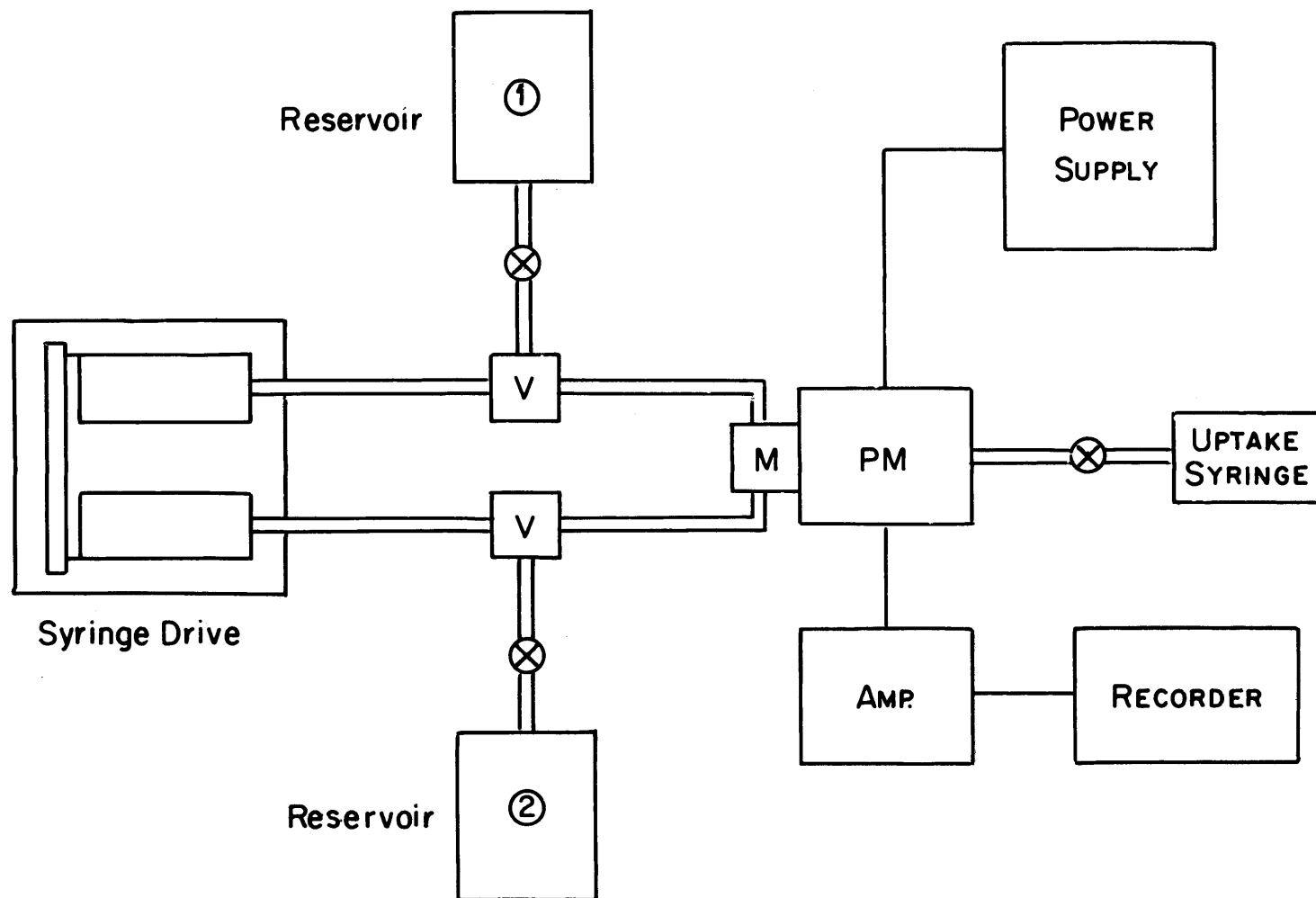


Table II-1

Syringe Drive Nominal Flow
(100 ml syringes)

<u>Speed</u>	<u>ml/min. per syringe</u>
1	77.4
2	55.3
3	39.5
4	28.2
5	20.2
12	1.9

Figure II-2

Teflon Mixer Used in Stopped-Flow Experiments

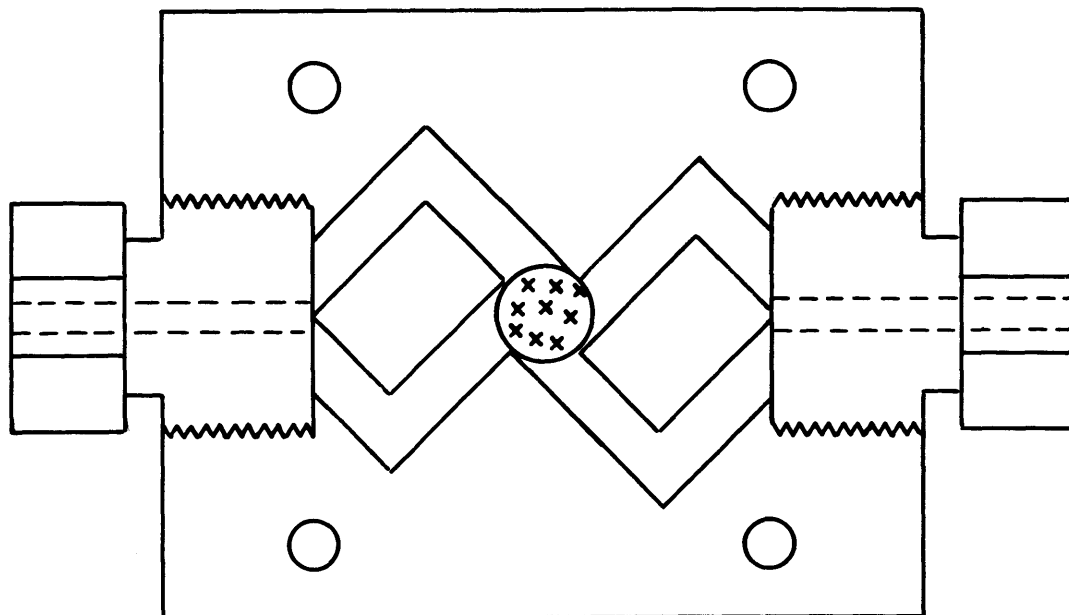
Key

xxx Platinum Gauze

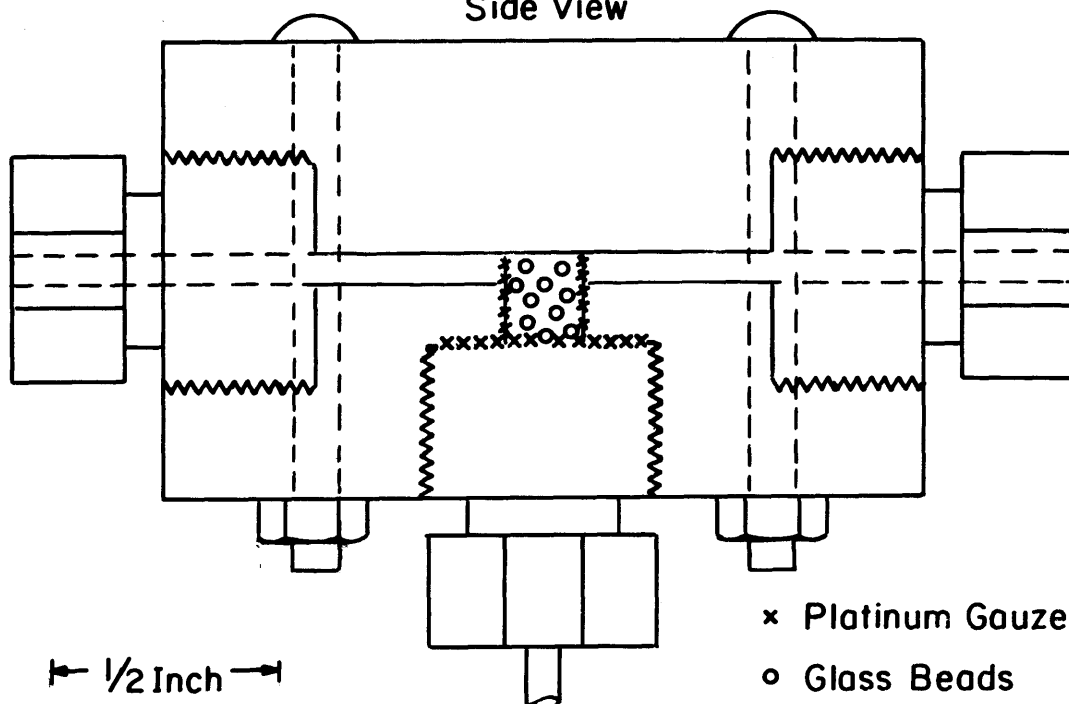
ooo Glass Beads

TEFLON MIXER

Top View



Side View



mounted on the stopcock was adjusted so that it shut off the syringe drive the moment the flow was stopped.

"Cheminert" three-way valves and fittings (Chromatronix, Inc.) and Teflon tubing were used to complete the flow system. The only surfaces the solutions came in contact with were made of Teflon or glass.

The total light from the chemiluminescent reaction was detected with an RCA 1P21 photomultiplier powered by a Kepco Model ABC Power Supply operated at 750 volts. After amplification by an operational amplifier with variable feedback resistors, the signal was recorded on a Moseley 7100B Strip Chart Recorder equipped with a Model 17501A plug-in unit (Hewlett-Packard).

2. Chemiluminescence Cell

The spectral distribution of the steady-state chemiluminescence produced by rapidly mixing the reactants in a flow system was recorded on the Turner Spectrofluorimeter (luminescence mode) using the Teflon mixing "T" (Swagelok #200-3) and the cell shown in Figure II-3.

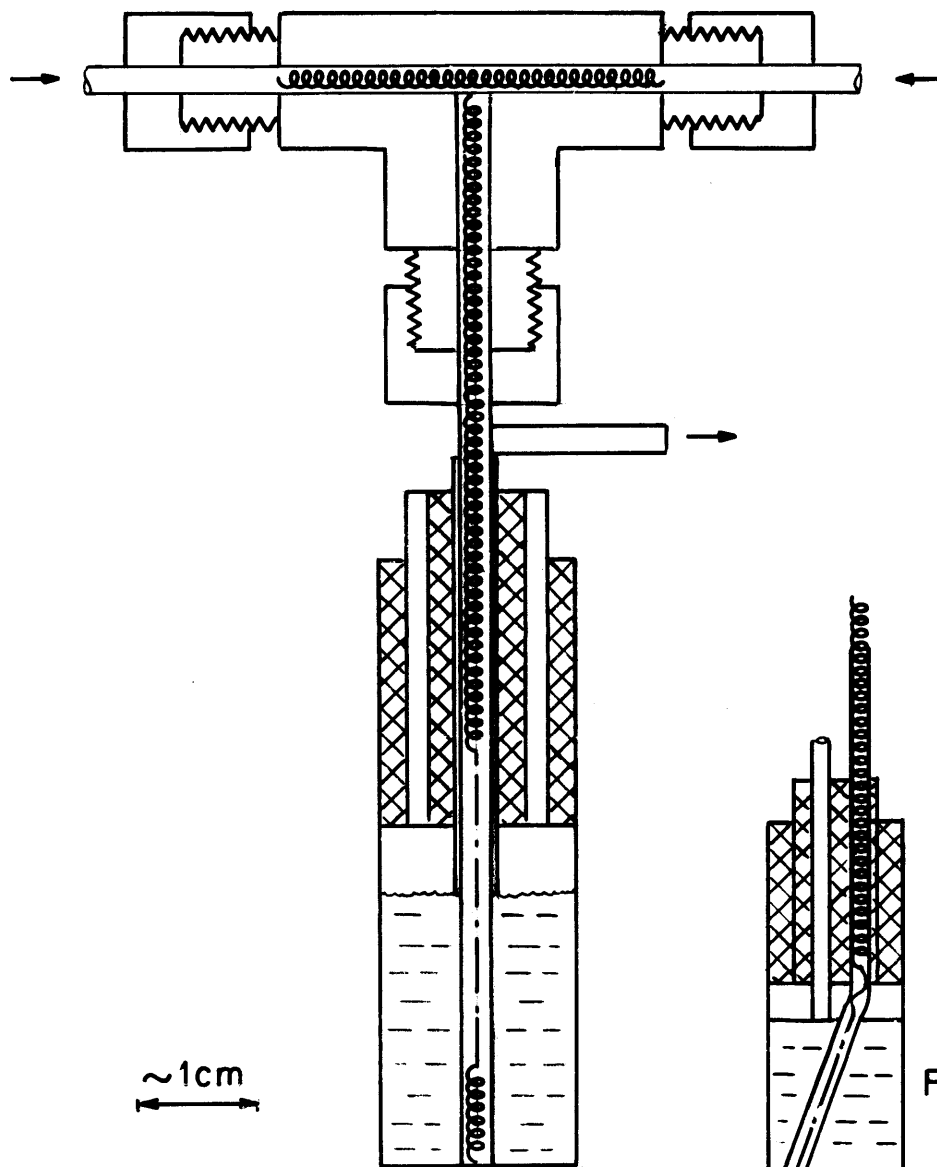
The CL cell was made from a 1.00 cm quartz cell with a square top. A one inch piece of 9 mm Pyrex tubing was inserted into the cell and held in place with RTV Silicone Rubber (General Electric). The 3 mm feeder and exit tubes were then positioned as shown and the cell sealed with RTV.

It was found that coils of platinum wire in the arms of

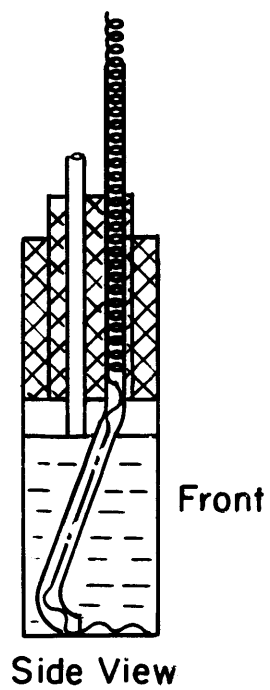
Figure II-3

CL Cell with Mixing "T"

CL CELL
WITH MIXING "T"



(Quartz cell
not to scale)



the "T" and down the feeder tube produced very rapid mixing.

3. Special Cell for Absorption and Fluorescence Spectra

Absorption and fluorescence spectra of luminol in basic DMSO and DMSO-water mixtures were obtained using the cell shown in Figure II-4. The design of the cell permitted complete vacuum degassing of the basic solvent before it was mixed with the luminol. A graded seal connected the 1.00 cm square quartz cell to the Pyrex neck. The degassing arm was a 9 cm Pyrex tube (11 mm O.D.) and held about 6 ml. The angle of the arm was such that the complete cell could be placed in the spectrophotometer and the spectrofluorimeter.

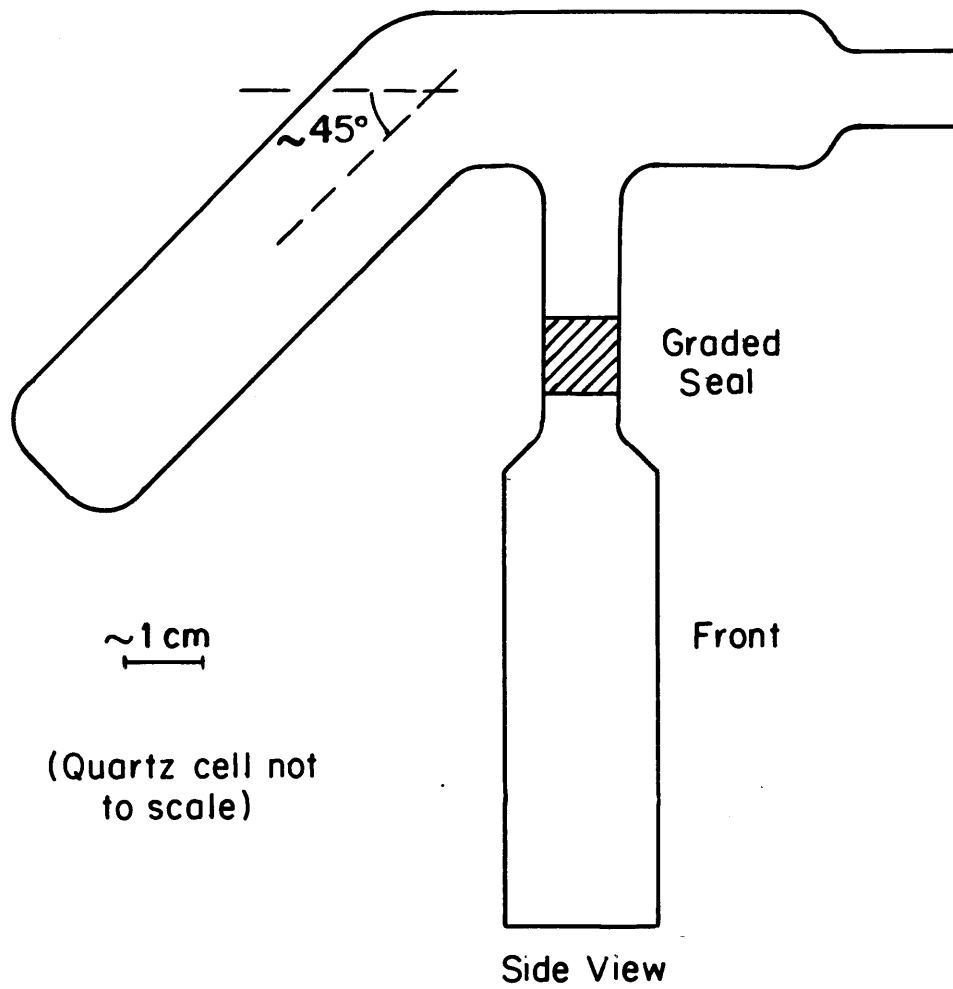
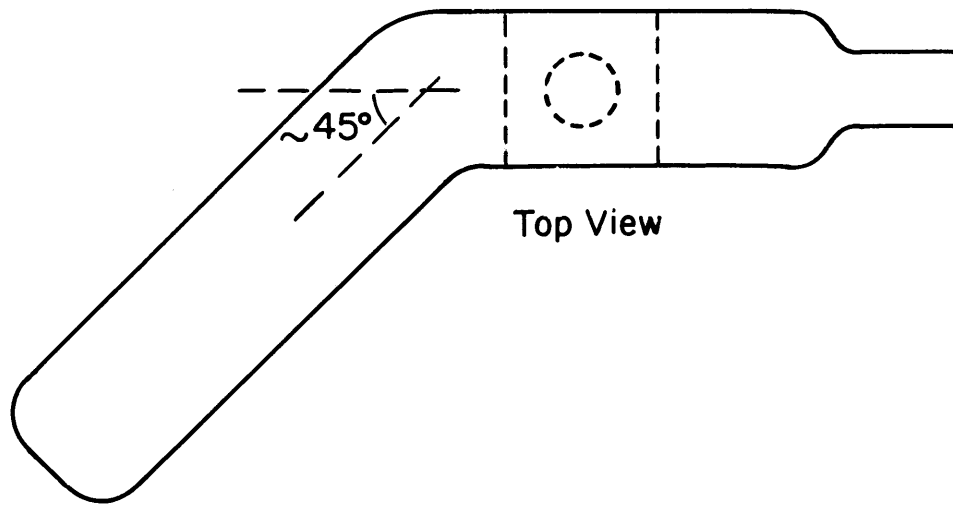
D. Special Procedures

1. Mixed Solvent Preparation

When preparing solutions of 3-APA and luminol in DMSO-water mixed solvents, special precautions had to be taken. Since heat is produced when DMSO and water are initially mixed together, the mixed solvents were allowed to cool to room temperature before use. The neutral mixtures were stable when stored in tightly sealed containers. The basic solvents were prepared by adding enough aqueous 1.0 N KOH to give the desired base concentration. When significant, the volume of base was considered as part of the total amount of water in the mixed solvent. The basic mixed solvents were found to decompose (even when degassed) after several hours, and therefore, the base was added just before use. This decomposition

Figure II-4

Special Cell for Absorption and Fluorescence
Spectra



of the DMSO by the base shifted the solvent ultraviolet cutoff to longer wavelengths.

2. Stopped-Flow Experiment

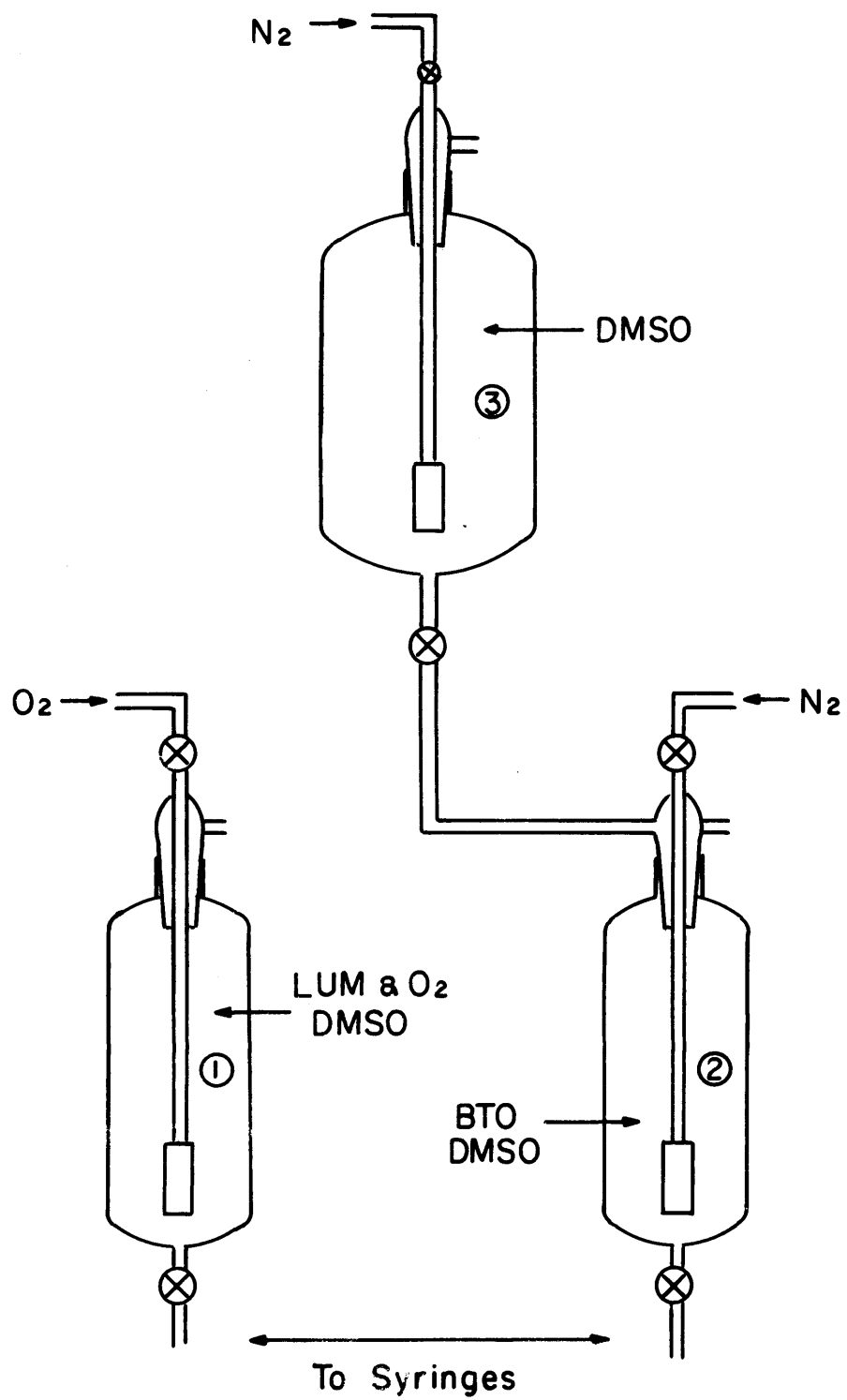
a. Solution Preparation

(i) DMSO

The stopped-flow experiments using DMSO required special precautions because BTO was used as the base. The BTO reacts rapidly with water to form KOH (which is insoluble in dry DMSO) and t-butyl alcohol. It also rapidly decomposes DMSO (solution turns yellow) unless the DMSO is thoroughly deoxygenated beforehand. A DMSO solution of BTO which was fairly stable for about an hour could be obtained using the apparatus shown in Figure II-5. This system was designed so that all the reservoirs and connections could be completely flushed with prepurified nitrogen. The nitrogen used for bubbling the solutions was presaturated with DMSO in a bubbling chamber containing DMSO over molecular sieves. Reservoir [2] (125 ml graduated or 500 ml separatory funnel depending on whether the base concentration was being varied or held constant, respectively) was placed in a dry, nitrogen filled glove bag which also contained an automatic balance. A sample of BTO was weighed on a platinum boat. After flushing the reservoir with nitrogen, the boat and sample were inserted into the reservoir, the stopcock was closed and the top sealed. This was put back into the apparatus as shown, flushed with nitrogen, and

Figure II-5

Apparatus for Solution Preparation
in Stopped-Flow Experiments



made up to volume by adding DMSO (dried over molecular sieves and bubbled with nitrogen for one hour) from reservoir [3] (500 ml separatory funnel). The base solution was bubbled with nitrogen throughout the experiment.

The above procedure was not used to prepare low concentrations of base because the BTO could not be weighed accurately. These solutions were prepared by successive dilutions of a stock BTO solution prepared as described above. After each dilution, about 20 ml of the new base solution were drawn into the syringe. This flushed the more concentrated solution from the syringe and tubing to the reservoir. This was discarded by forcing the solution through the mixer to the uptake syringe. The new base solution was drawn into the syringe until the reservoir contained the amount of solution needed for the next dilution. In general, about 40-50 ml were sufficient for a stopped-flow experiment.

All the kinetic experiments were run using NaLUM. These solutions were prepared beforehand and transferred to reservoir [1] (125 or 500 ml separatory funnel depending on whether the luminol concentration was being varied or held constant, respectively) in Figure II-5.

Since the base solution must be oxygen free, the NaLUM solution was the carrier of the oxygen necessary for the chemiluminescent reaction. The amount of oxygen in solution was varied by bubbling various mixtures of oxygen and nitrogen (DMSO presaturated) through the NaLUM solution for about 30 minutes.

The ratio of oxygen to nitrogen was monitored with calibrated flow-meters and the concentration of oxygen was calculated assuming that the solubility of oxygen in DMSO is about $2 \times 10^{-3} \text{ M}$ (27) (28).

(ii) Mixed Solvents

The best procedure for ensuring that the NaLUM and base solutions had the same mixed solvent composition was to prepare each solution from a separate mixed solvent stock. A neutral mixed solvent with the required amount of water was prepared as described above and used to dilute a concentrated stock solution of NaLUM in DMSO ($\sim 6 \times 10^{-3} \text{ M}$). This dilution does not significantly change the solvent composition because the solvents of particular interest in this study contained mostly DMSO and the dilutions were never greater than 5 ml stock to 100 ml mixed solvent. This NaLUM solution ($\sim 2 \times 10^{-4} \text{ M}$) was then saturated with oxygen.

Another neutral mixed solvent which contained the same amount of water less the amount of aqueous 1.0 N KOH to be added was prepared and bubbled with nitrogen for 30 minutes. The base was then added, and the basic mixed solvent was bubbled with nitrogen an additional 10 minutes before use.

b. Stopped-Flow Run

For a typical stopped-flow experiment, the entire apparatus was flushed with nitrogen and the solutions prepared as described above. The solutions for the run were in reservoirs [1]

and [2] (see Figure II-1). The three-way valves were opened to the reservoirs and about 20 ml of solution were drawn into each syringe. The syringes were then removed from the syringe drive, inverted and the nitrogen which was in the lines to the reservoirs was forced back through the solution in the reservoirs until there were no more bubbles. The syringes were filled to about 50 ml and locked in the syringe drive. The syringe drive was set to speed #1, turned on, and run until the drive plate came in contact with both syringes and the solution was being driven back into the reservoirs. This was important because if both syringes were not in contact with the drive plate and flow was directed to the mixer, solution from one syringe was forced into the other syringe, and the experiment was ruined. The valves were then set to direct flow to the mixer. The syringe drive was turned on again, and the mixer, observation tube, and tubing to the uptake syringe were filled with solution. A flow slower than obtained with speed #1 was used to remove all the bubbles. Speed #1 was again selected and a trial run was made to observe the light intensity and approximate decay time so that the recorder range and chart speed could be selected. For a quantitative run the chart drive was switched on, the stopcock on the uptake syringe was opened (also starting the syringe drive), and when a steady-state light level was reached, the flow and syringe drive were quickly stopped by closing the stopcock. The CL intensity was then monitored as a function of time. Several runs were recorded for each experiment, and the resulting decay curves were very reproducible when the

solutions were freshly prepared and the base solutions thoroughly deoxygenated. There was some decrease in light intensity when the syringes were about empty due to decomposition of the base solution.

3. Spectra of Luminol in Basic Solution

These spectra were very difficult to obtain because the basic solutions of NaLUM must be completely deoxygenated. It was found that bubbling with prepurified nitrogen was not sufficient to prevent basic solutions of NaLUM in DMSO and some of the mixed solvents from chemiluminescing. A solution which was prepared by adding a weighed sample of NaLUM to a degassed solvent in a dry, nitrogen filled glove bag chemiluminesced because of the small amount of oxygen which was introduced with the NaLUM sample. The cell shown in Figure II-4 allowed complete vacuum degassing of both the basic solvent and the NaLUM before they were mixed. The same volume (0.2 ml) of a stock solution of NaLUM in DMSO (3.0×10^{-3} M) was used to obtain all the spectra in this study.

a. Mixed Solvents

The basic mixed solvents (2.5×10^{-3} N KOH) were prepared as described above. Appropriate hypodermic syringes were used to inject 0.2 ml of the stock luminol solution and 5.0 ml of the basic solvent into the quartz cell and Pyrex tube, respectively. The cell was connected to a vacuum system with butyl rubber tubing, and the solvent frozen in liquid nitrogen. The DMSO was vacuum

evaporated from the luminol solution and trapped in the cold Pyrex tube with the solvent. (The small amount of additional DMSO in the mixed solvent does not significantly change its composition.) When the DMSO was evaporated, the mixed solvent (5.2 ml) was vacuum degassed four times. A slight positive pressure of helium was put into the cell while the solvent was still frozen. The cell was sealed by clamping the rubber tubing and removed from the vacuum system. The solvent was then thawed and mixed with the luminol in the quartz cell. Absorption and fluorescence spectra were recorded as rapidly as possible. The reference cell for the absorption spectra contained the basic mixed solvent and was deoxygenated by bubbling with nitrogen for 20 minutes. After these spectra were obtained, the cell was opened and bubbled with oxygen until no more CL was observed. The cell was then degassed with nitrogen for 15 minutes. Absorption, fluorescence, and excitation spectra of the reaction product were recorded. No CL was observed from the NaLUM solutions which were prepared from the basic (2.5×10^{-3} N KOH) mixed solvents containing greater than 30 mole % water. These solutions were deoxygenated by bubbling with DMSO-presaturated nitrogen for 30 minutes.

b. DMSO

Absorption, fluorescence, and excitation spectra of NaLUM in DMSO containing various amounts of freshly sublimed BTO were obtained by modifying the above procedure. The NaLUM stock (0.2 ml) was added to the quartz cell, and the DMSO was vacuum

evaporated and condensed in a cold trap outside the cell. The cell was removed from the vacuum system and 5.2 ml of DMSO were injected into the Pyrex tube. The solvent was vacuum degassed four times, and helium was put into the cell. While the DMSO was still frozen, the cell was quickly opened, and a small amount of BTO (samples weighed under nitrogen or several microliters of a stock solution ($\sim 10^{-1}$ M) which was kept frozen between additions) was placed in the neck of the cell. The DMSO was vacuum degassed two more times and helium put into the cell again. The DMSO was thawed and rapidly mixed with the BTO and NaLUM. Absorption, fluorescence, and excitation spectra were recorded as quickly as possible. The base concentration was changed by adding various amounts of BTO to the cell. When the stock BTO was used, the very small amount of DMSO introduced with the sample was evaporated during the last freeze-thaw cycles. Solvent decomposition occurred very rapidly when an excess of BTO was present. However, very "clean" absorption spectra were obtained by preparing a new luminol solution before each addition of base and recording the spectra immediately after mixing the components.

The reference cell contained DMSO which had been deoxygenated by bubbling with nitrogen for 30 minutes.

Several of the solutions were allowed to react with oxygen, and the reaction product absorption, fluorescence, and excitation spectra were recorded. The absorption spectra were very distorted because of solvent decomposition.

4. Chemiluminescence Spectra

The solution preparation was the same as that described for the stopped-flow experiments. The syringe drive was used to provide a continuous flow through the Teflon tubing to the mixing "T" and special CL cell (Figure II-3) which was positioned in the cell compartment of the Turner Model 210 Spectrofluorimeter. For some of the mixed solvent experiments the same solutions were used to record both the CL decay curve and the CL spectrum. This was done by modifying the apparatus so that the flow could be directed either to the four-jet mixer or the CL cell. The spectrofluorimeter was used in the luminescence mode with the lamp turned off. In this mode the instrument records corrected spectra in relative quanta per unit bandwidth. The cell was first flushed with the mixed solution using speed #2 or #3 on the syringe drive. This removed bubbles from the system and allowed adjustment of the instrument (emission slit width, range, etc.) for the light level of the reaction. The syringe drive was then changed to speed #12, and when a steady-state light level was obtained, several CL spectra were recorded. These were found to be very reproducible. After the last CL spectrum was recorded, the flow was stopped, and the reaction was allowed to go to completion. Fluorescence and excitation spectra of the reaction product were recorded.

III. Results

A. Luminol Study

1. Absorption and Fluorescence Spectra of Luminol

a. Spectra in Aqueous Solution

The absorption spectra of luminol in aqueous solution as a function of pH are shown in Figure III-1. Absorption and fluorescence data are tabulated in Table III-1. At pH \approx 0 the amino group is protonated (λ_{max} = 263, 298 nm) and, as expected, the spectrum is similar to the PD absorption spectrum (see Section III-A-5). Increasing the pH to 4.5 shifts the equilibrium (isosbestic points at 256, 267, 308, and 319 nm) to the molecular species (λ_{max} = 294, 350 nm). At pH = 11.2 luminol ($\text{pK}_a \approx 6.5$) exists as the mononegative ion (λ_{max} = 301, 348 nm; isosbestic points at 290 nm and 371 nm). The changes observed in the absorption spectrum at pH \approx 14 could be due to the formation of a very small amount of a new species, possibly the dinegative ion. The formation of a new species is supported by the fact that two fluorescence bands (λ_{max} = 420, 505 nm; about equal intensity) are also observed. Excitation spectra for these bands (λ_{em} = 400, 560 nm, respectively) were very similar to each other and the absorption spectrum of the mononegative ion. Three different samples of luminol showed the same behavior.

Figure III-1

Absorption Spectra of Luminol
in Aqueous Solution

$[C] = 1.1 \times 10^{-4} M$; 1.00 cm cell

Key

— — — — —	1.0 <u>N</u> HCl
- - - - -	HAc-NaAc Buffer (pH = 4.5)
- - - - -	$2.5 \times 10^{-3} \underline{N}$ KOH
—————	1.0 <u>N</u> KOH

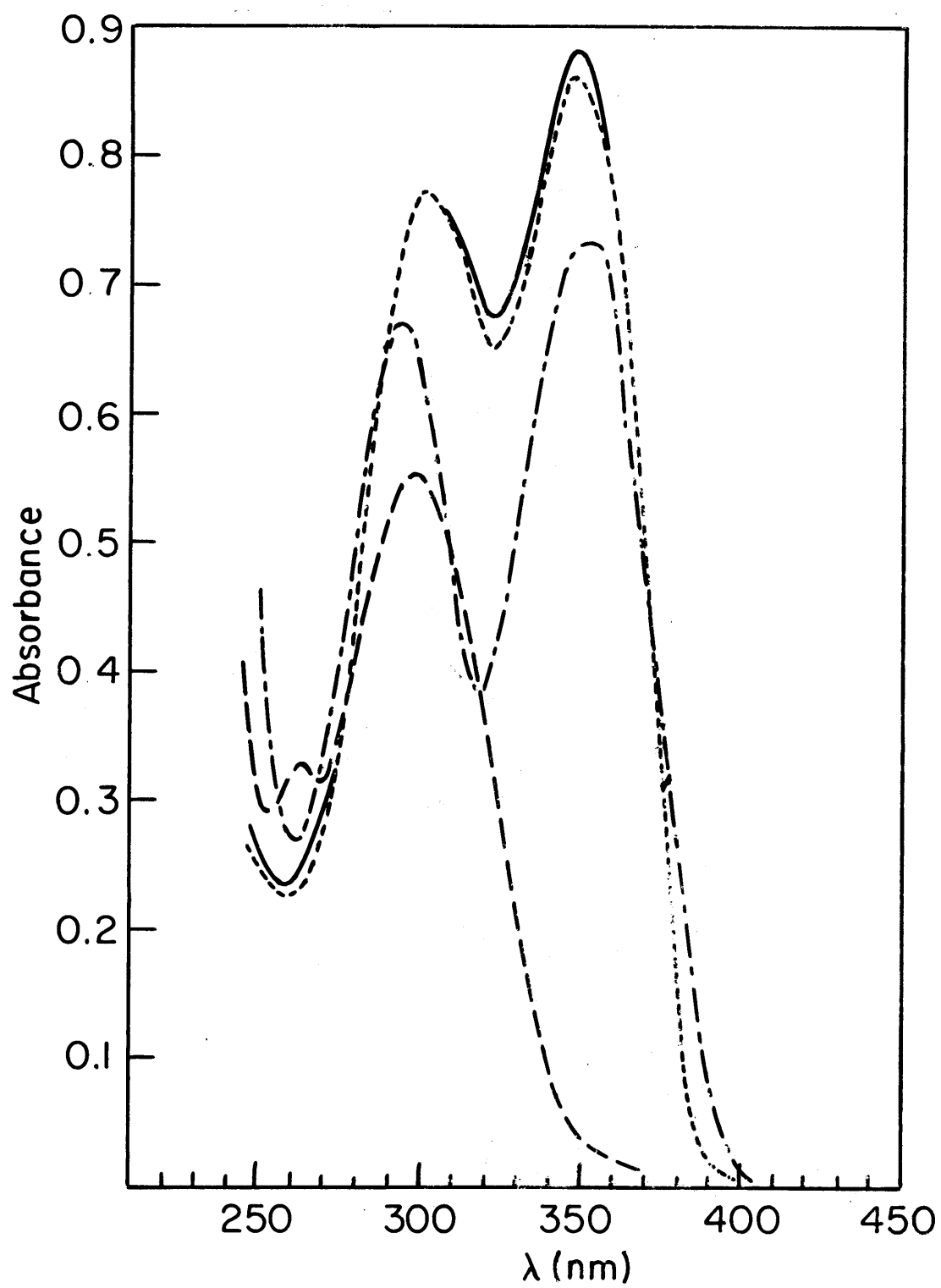


Table III-1

Absorption and Fluorescence Characteristics of Luminol
in Aqueous Solution as a Function of pH

<u>pH</u>	Absorption <u>$\lambda_{\max}(\text{nm}), (\epsilon)$</u>	Fluorescence <u>$\lambda_{\max}(\text{nm})$</u>
~0	263, (2900); 298, (4800)	475
4.5	294, (5850); 350, (6410)	430
11.2	301, (6700); 348, (7520)	410
~14	302; 348	420, 505 ^a

a- about equal intensity

b. Spectra in DMSO

Absorption spectra of luminol ($\lambda_{\text{max}} = 297, 360 \text{ nm}$), the mononegative ion ($\lambda_{\text{max}} = 333, 370 \text{ nm}$), and a species believed to be the dinegative ion ($\lambda_{\text{max}} = 310, 393 \text{ nm}$) in DMSO are shown in Figure III-2. Absorption spectra of vacuum-degassed solutions of NaLUM in DMSO as a function of base (BTO) concentration established the monoanion-dianion equilibrium and showed that about 10^{-3} M BTO was sufficient to completely shift the equilibrium to the dianion. Isosbestic points for the equilibria are 265, 312, 343, 371 nm, and 290, 378 nm, respectively.

Extinction coefficients and fluorescence data are found in Table III-2. Although the fluorescence quantum yields, ϕ_f 's, were not measured, the dianion appeared to have a greater ϕ_f than the monoanion. For each species the excitation spectrum was similar to the absorption spectrum.

The monoanion was not chemiluminescent. CL was observed only when solutions containing some of the dianion (determined by absorption spectrum) were exposed to oxygen.

c. Spectra in DMSO-Water Mixed Solvents

Figures III-3, III-4, III-5, and III-6 show the absorption spectra of NaLUM in various basic ($2.5 \times 10^{-3} \text{ N KOH}$) DMSO-water mixed solvents. The spectra of NaLUM in aqueous $2.5 \times 10^{-3} \text{ N KOH}$ and basic DMSO are also included. These spectra are grouped according

Figure III-2

Absorption Spectra of Luminol
in Neutral and Basic DMSO

$[C] = 1.1 \times 10^{-4} M$; 1.00 cm cell

Base = BTO

Key

— — — — —	Luminol
- - - - -	Luminol Monoanion
- - - - -	Luminol Dianion

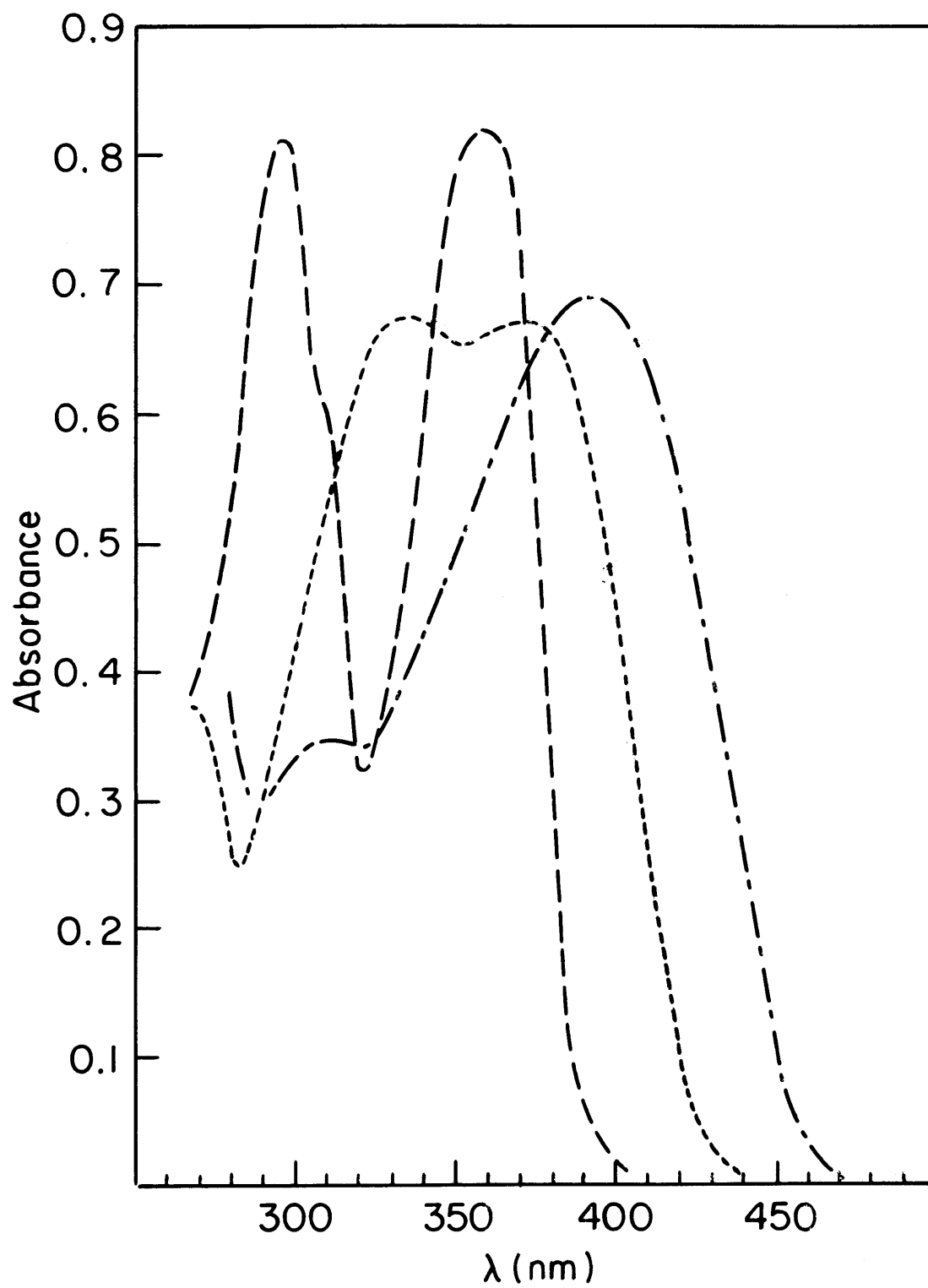


Table III-2

Absorption and Fluorescence Characteristics of Luminol,
Luminol Monoanion, and Luminol Dianion in DMSO

<u>Compound</u>	<u>Absorption</u> <u>λ_{max}(nm), (ϵ)</u>	<u>Fluorescence</u> <u>λ_{max}(nm)</u>
Luminol	297, (7650); 360, (7720)	410
Monoanion ^a	333, (6370); 370, (6320)	500
Dianion ^b	310, (3250); 393, (6500)	520

a- NaLUM with a very small amount of BTO added

b- BTO used as the base

Figure III-3

Absorption Spectra of NaLUM in Basic DMSO and Basic DMSO-Water

Mixed Solvents 1, 3, and 4

$[C] = 1.1 \times 10^{-4}M$; 1.00 cm cell

Key

— — — — —	Basic DMSO (BTO)
- - - - -	Mixed Solvent 1
- - - - -	Mixed Solvent 3
—————	Mixed Solvent 4

Base = $2.5 \times 10^{-3}N$ N KOH

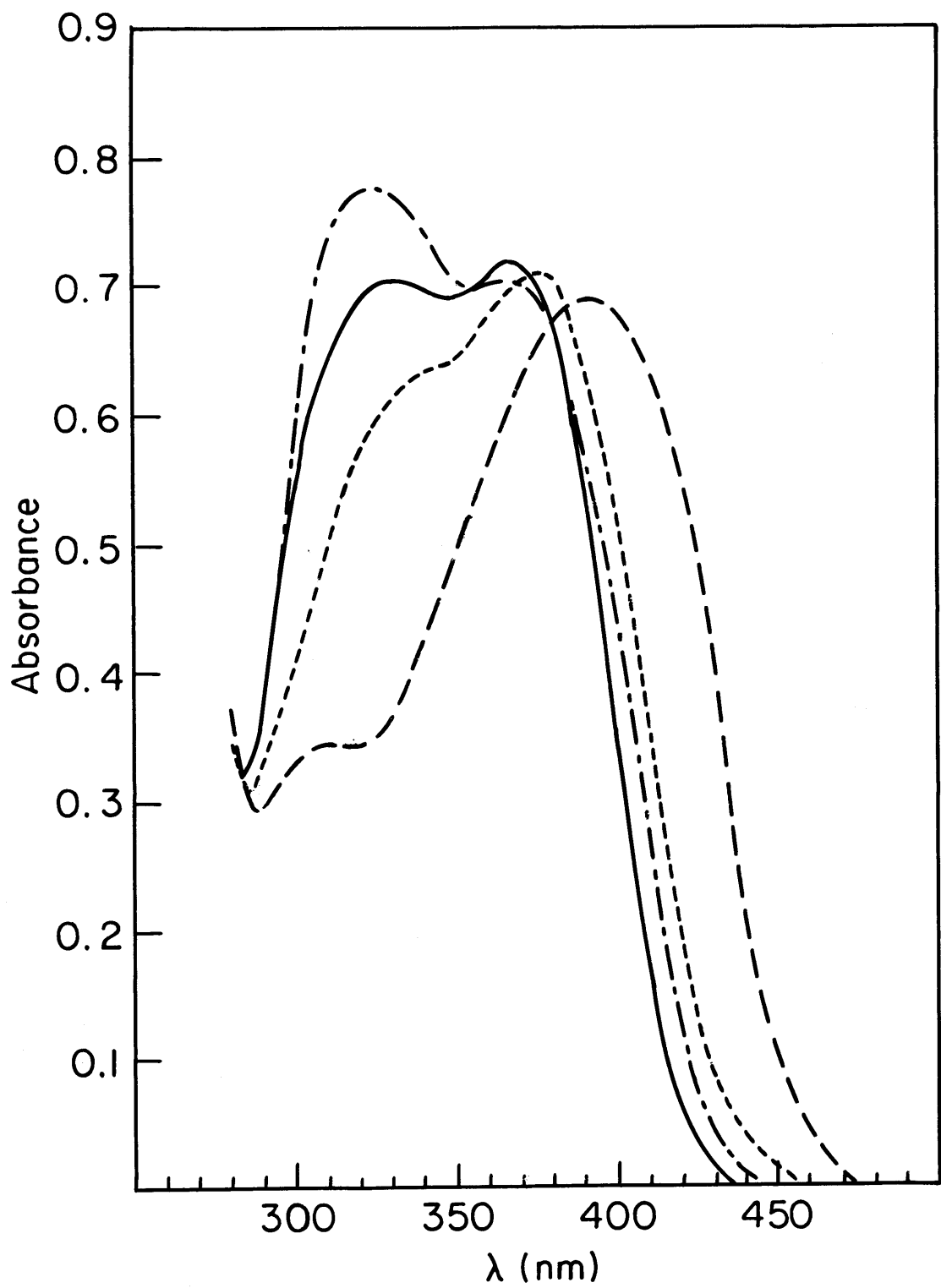


Figure III-4

Absorption Spectra of NaLUM in Basic DMSO-Water

Mixed Solvents 4, 5, and 6

$[C] = 1.1 \times 10^{-4} M$; 1.00 cm cell

$2.5 \times 10^{-3} N$ KOH

Key

—————	Mixed Solvent 4
- - - - -	Mixed Solvent 5
- - - - -	Mixed Solvent 6

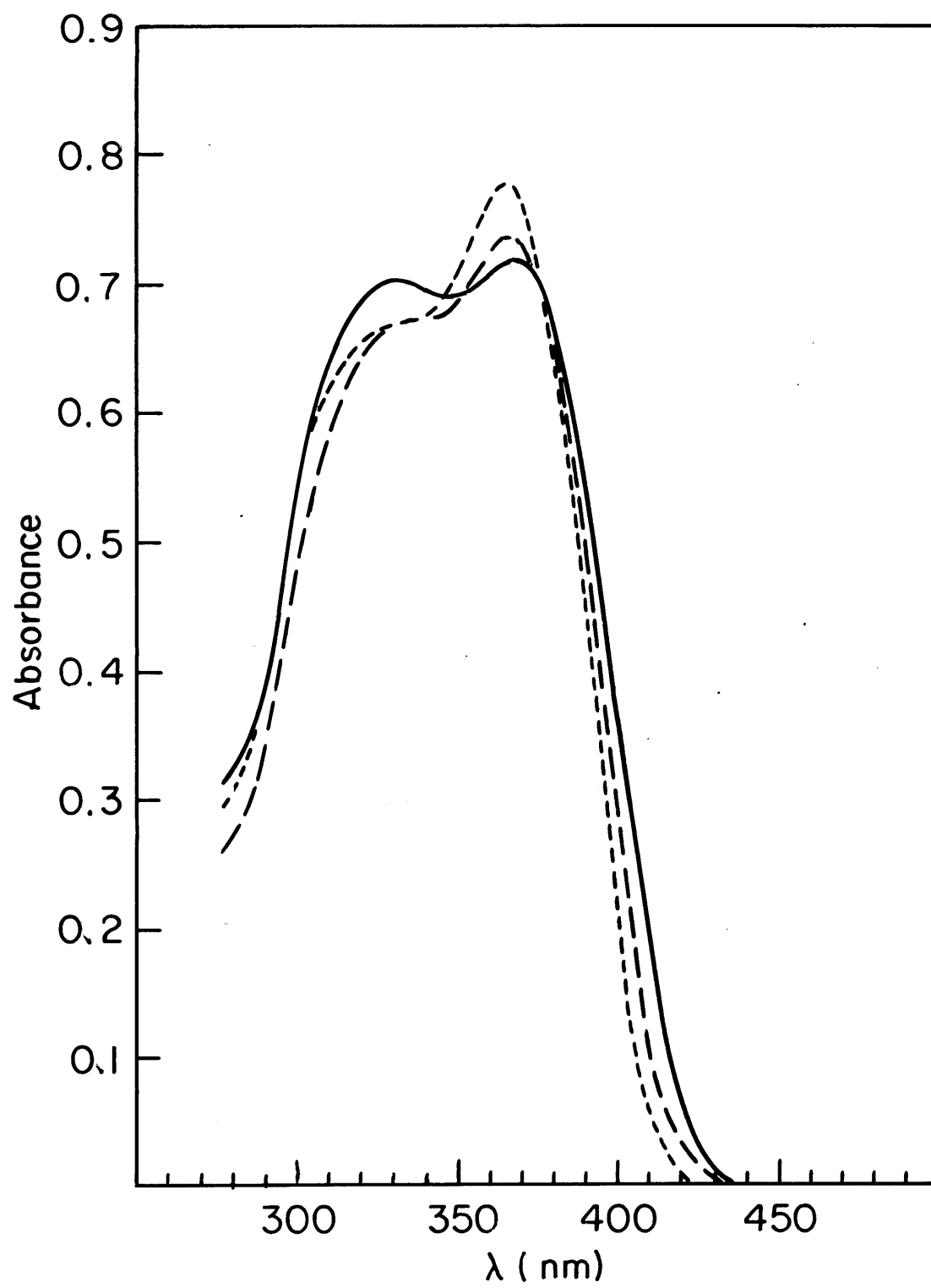


Figure III-5

Absorption Spectra of NaLUM in Basic DMSO-Water

Mixed Solvents 6, 8, 9, and 10

$[C] = 1.1 \times 10^{-4} M$; 1.00 cm cell

$2.5 \times 10^{-3} N$ KOH

Key

-----	Mixed Solvent 6
-----	Mixed Solvent 8
-----	Mixed Solvent 9
-----	Mixed Solvent 10

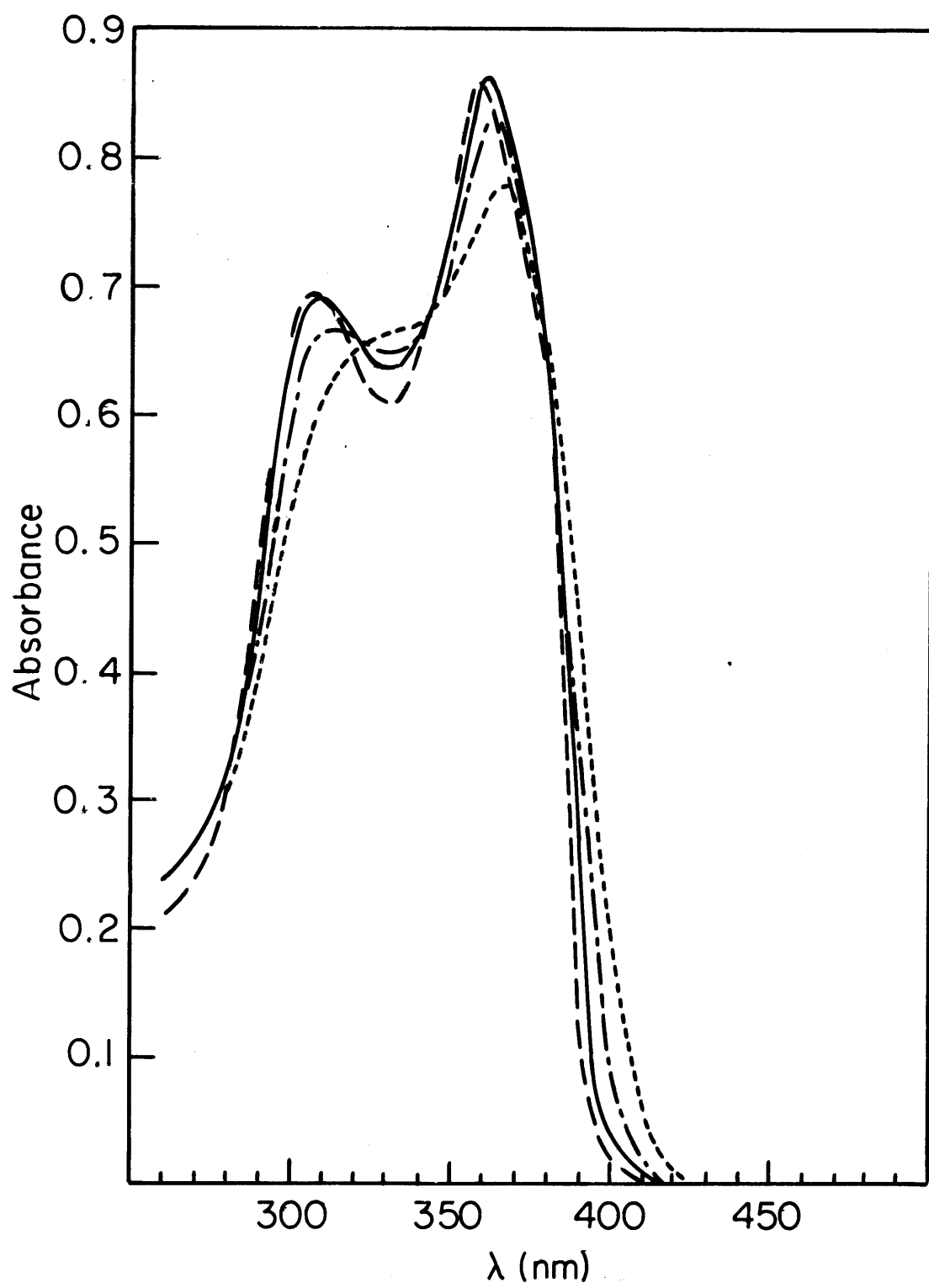


Figure III-6

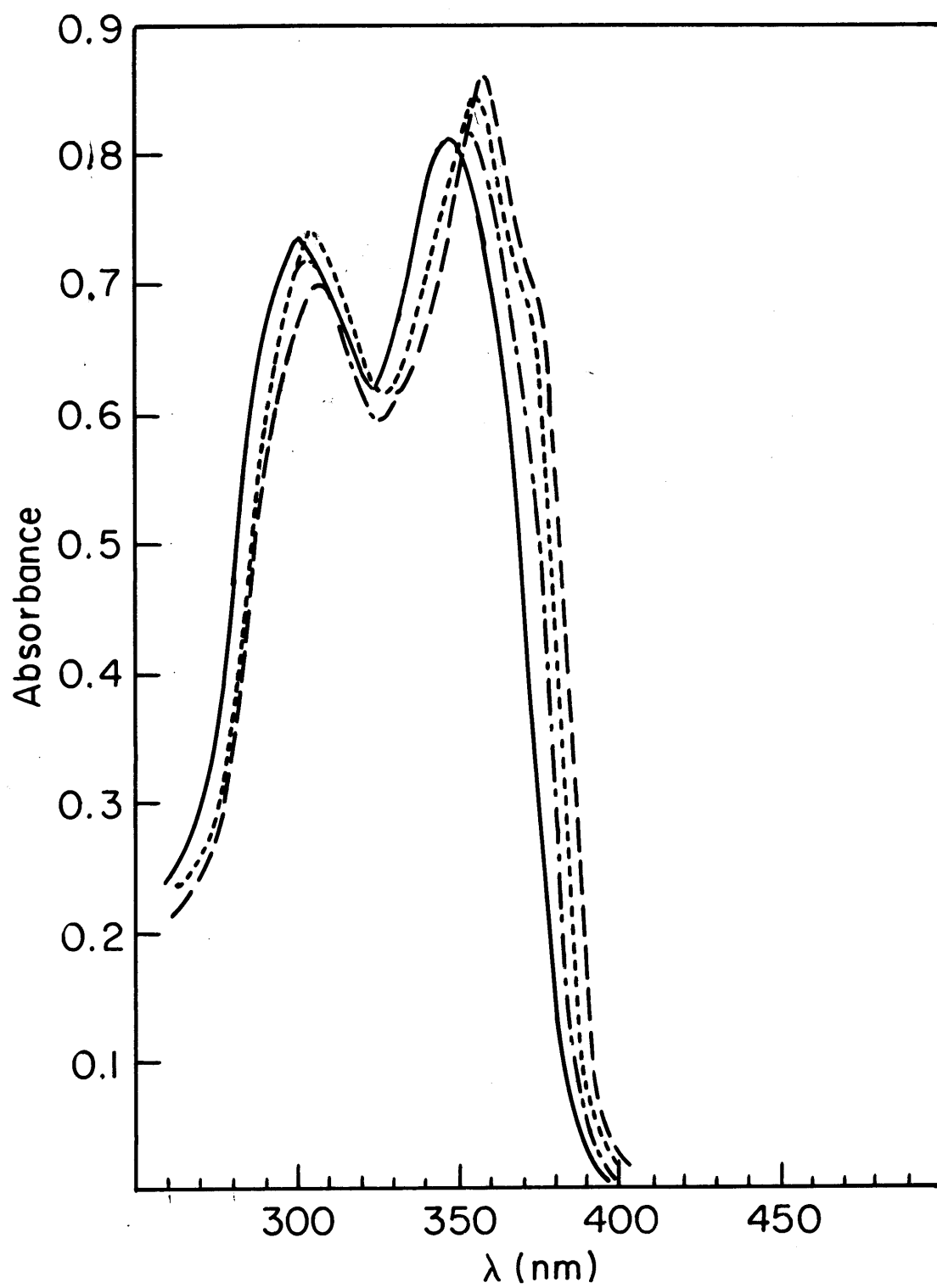
Absorption Spectra of NaLUM in Basic DMSO-Water
Mixed Solvents 10, 11, and 12 and Aqueous Base

$[C] = 1.1 \times 10^{-4} M$; 1.00 cm cell

$2.5 \times 10^{-3} N$ KOH

Key

— — — — —	Mixed Solvent 10
- - - - -	Mixed Solvent 11
— - - - —	Mixed Solvent 12
—————	Aqueous Base



to the different spectral shifts observed with increasing amounts of water. For the purpose of comparison, each figure contains the highest mole % water spectrum from the preceding figure. The compositions of the mixed solvents are given in Table III-3 and will be referred to by number.

Very small amounts of water (Figure III-3; mixed solvents 1 and 3) rapidly decrease the concentration of the dinegative ion and cause the spectra to shift to shorter wavelengths with increased absorption in the 320-330 nm region. The spectra for mixed solvents 4 and 5 (Figure III-4) show increased absorption around 365 nm at the expense of the short wavelength band. Higher concentrations of water (Figure III-5; mixed solvents 6, 8, 9, and 10) show what seems to be an equilibrium in which a new band appears at 310 nm while the 365 nm band increases in intensity and shifts to 360 nm. The spectra for mixed solvents 10, 11, and 12 in Figure III-6 show the solvent shift which leads to the mononegative ion spectrum in aqueous base.

The fluorescence spectra of luminol in the basic mixed solvents are complex. In general, the fluorescence maximum gradually shifts from 520 nm to 410 nm as the amount of water is increased. Excitation spectra for mixed solvents 1 and 3 showed that more than one species was responsible for the emission. For mixed solvent 1 the excitation spectrum obtained using the emission at 600 nm was similar to the dinegative ion absorption spectrum. Excitation spectra for mixed solvents 4-12 were very similar to the

Table III-3

Mixed Solvent Compositions

DMSO-Water

<u>Number</u>	<u>ml (H₂O)</u>	<u>ml (DMSO)</u>	<u>Mole % H₂O</u>	<u>Mole % DMSO</u>
1	0.25	100.0	1	99
2	0.50	100.0	2	98
3	1.0	100.0	4	96
4	2.5	100.0	9	91
5	5.0	100.0	16	84
6	10.0	100.0	28	72
7	15.0	85.0	41	59
8	20.0	80.0	50	50
9	30.0	70.0	63	37
10	40.0	60.0	72	28
11	60.0	40.0	86	14
12	80.0	20.0	94	6

absorption spectra of NaLUM in these solvents.

Solutions of NaLUM in basic ($2.5 \times 10^{-3} \text{ N KOH}$) mixed solvents 1-5 chemiluminesce when bubbled with oxygen. However, the CL intensity falls off quite dramatically with increasing amounts of water (see Section III-A-2).

The absorption spectra of luminol in neutral DMSO, water (pH = 4.5), and neutral mixed solvents 8, 10, and 12 are shown in Figure III-7. The absorption spectra for mixed solvents 1-7 are identical to the DMSO spectrum. The shifts in the luminol absorption spectrum with increasing amounts of water are very similar to the spectral changes observed for the NaLUM absorption spectrum in mixed solvents 10, 11, and 12 (see Figure III-6).

2. Luminol CL Spectra in DMSO and DMSO-Water Mixed Solvents

The CL spectra of luminol in DMSO and mixed solvents 1, 2, and 3 are identical ($\lambda_{\text{max}} = 482, 495 \text{ nm}$; referred to as 490 nm). The spectrum is shown in Figure III-8. Higher concentrations of water result in the appearance of a new band in the CL spectrum at shorter wavelengths ($\sim 410\text{-}430 \text{ nm}$). Figure III-9 shows the normalized CL spectra obtained using mixed solvents 4, 5, 6, and 7.

The spectra in mixed solvents 1 and 2 were obtained using a $2.5 \times 10^{-4} \text{ M NaLUM}$ solution saturated with oxygen, $1.3 \times 10^{-3} \text{ N KOH}$ and a 25A emission slit. For mixed solvents 3, 4, and 5 a base concentration of $2.5 \times 10^{-3} \text{ N KOH}$ was used and the slit had to be changed from 25A to 100A and 250A for mixed solvents 4 and 5,

Figure III-7

Absorption Spectra of Luminol in DMSO, Water
and Neutral DMSO-Water Mixed Solvents

$[C] = 9.0 \times 10^{-4} M$; 1.00 cm cell

Key

— — — — —	DMSO
- - - - -	Mixed Solvent 8
- - - - -	Mixed Solvent 10
—————	Mixed Solvent 12
- - - - -	Water (pH = 4.5)

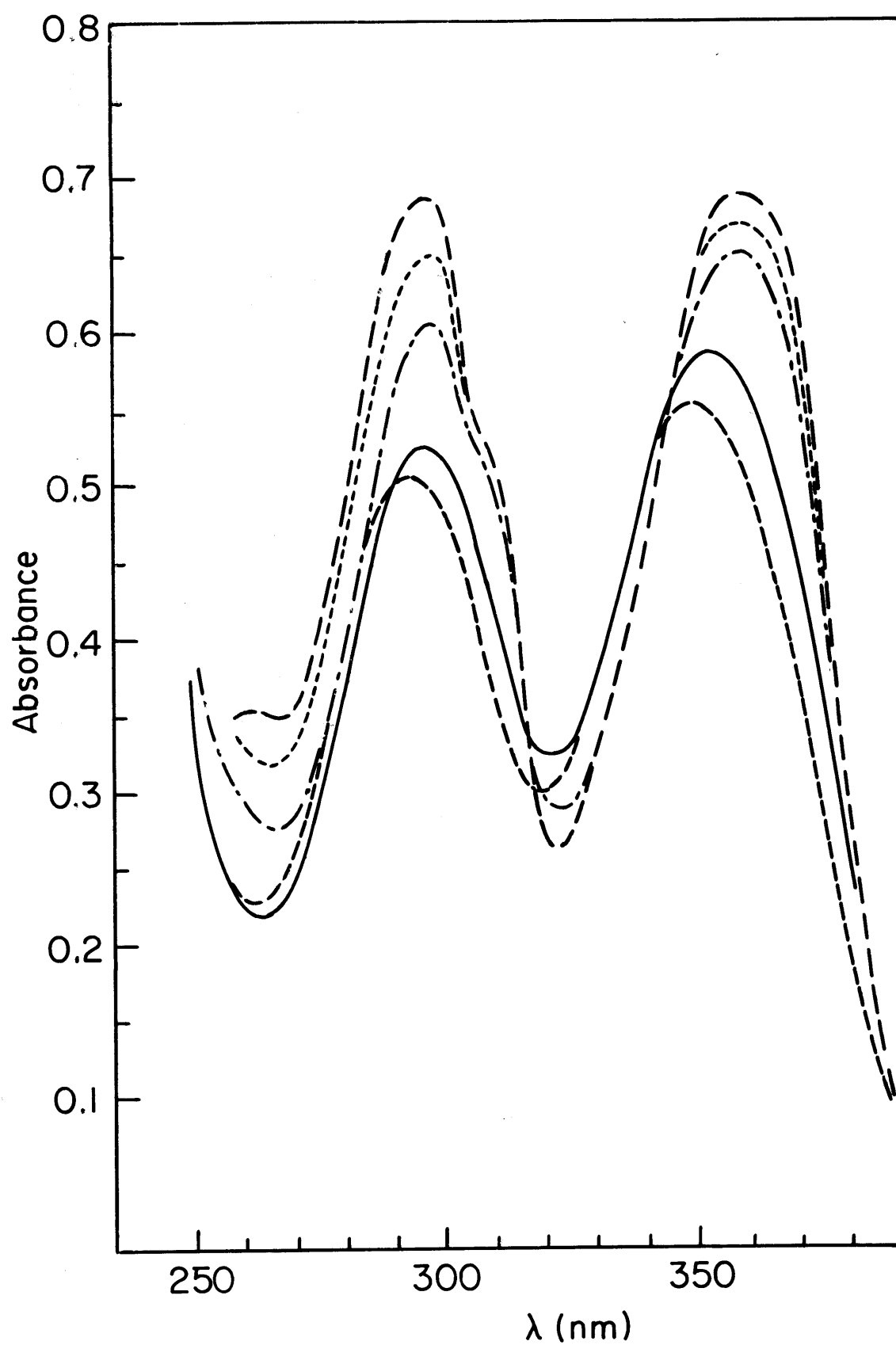


Figure III-8

Luminol CL Spectrum in DMSO and DMSO-Water

Mixed Solvents 1, 2, and 3

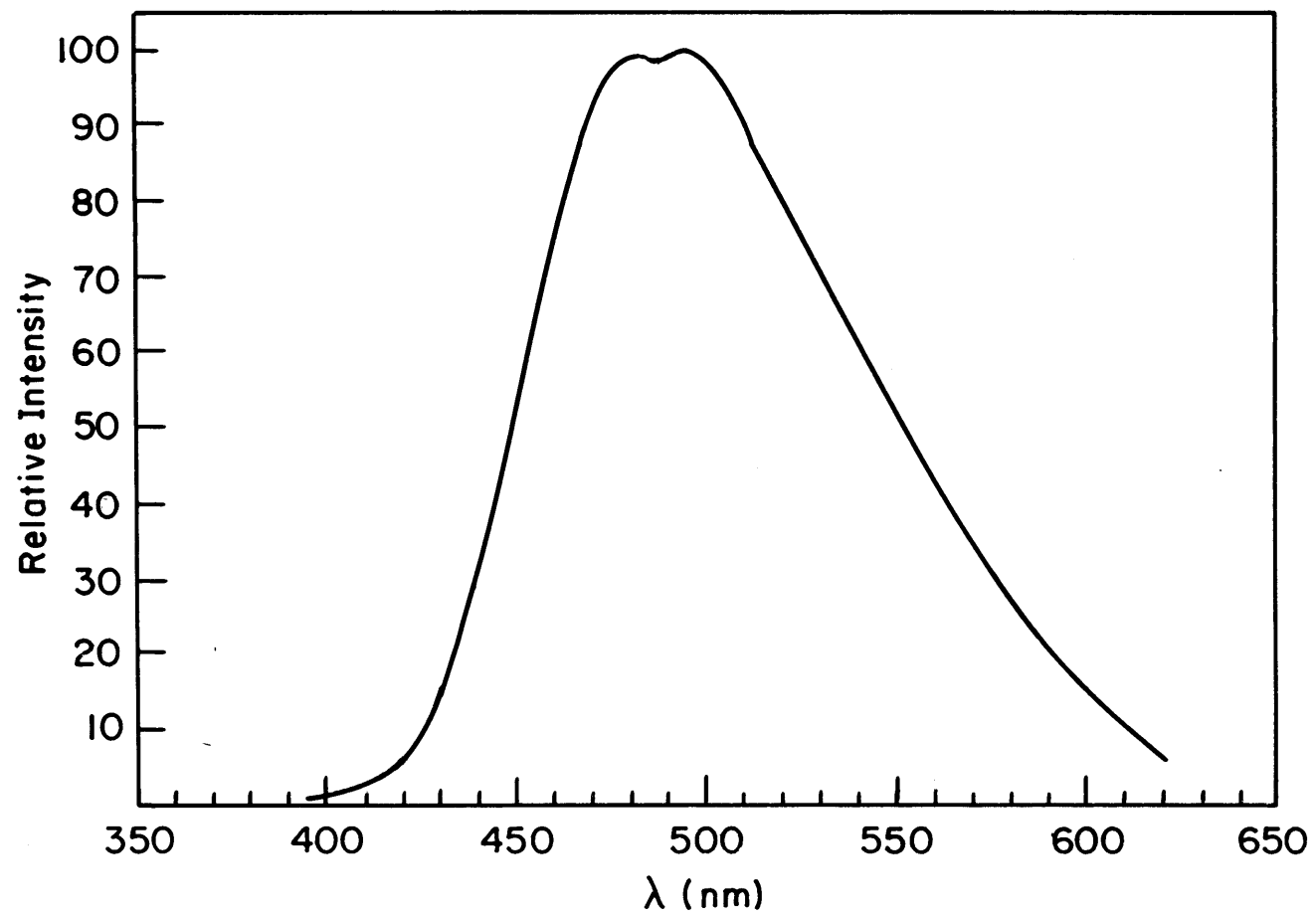
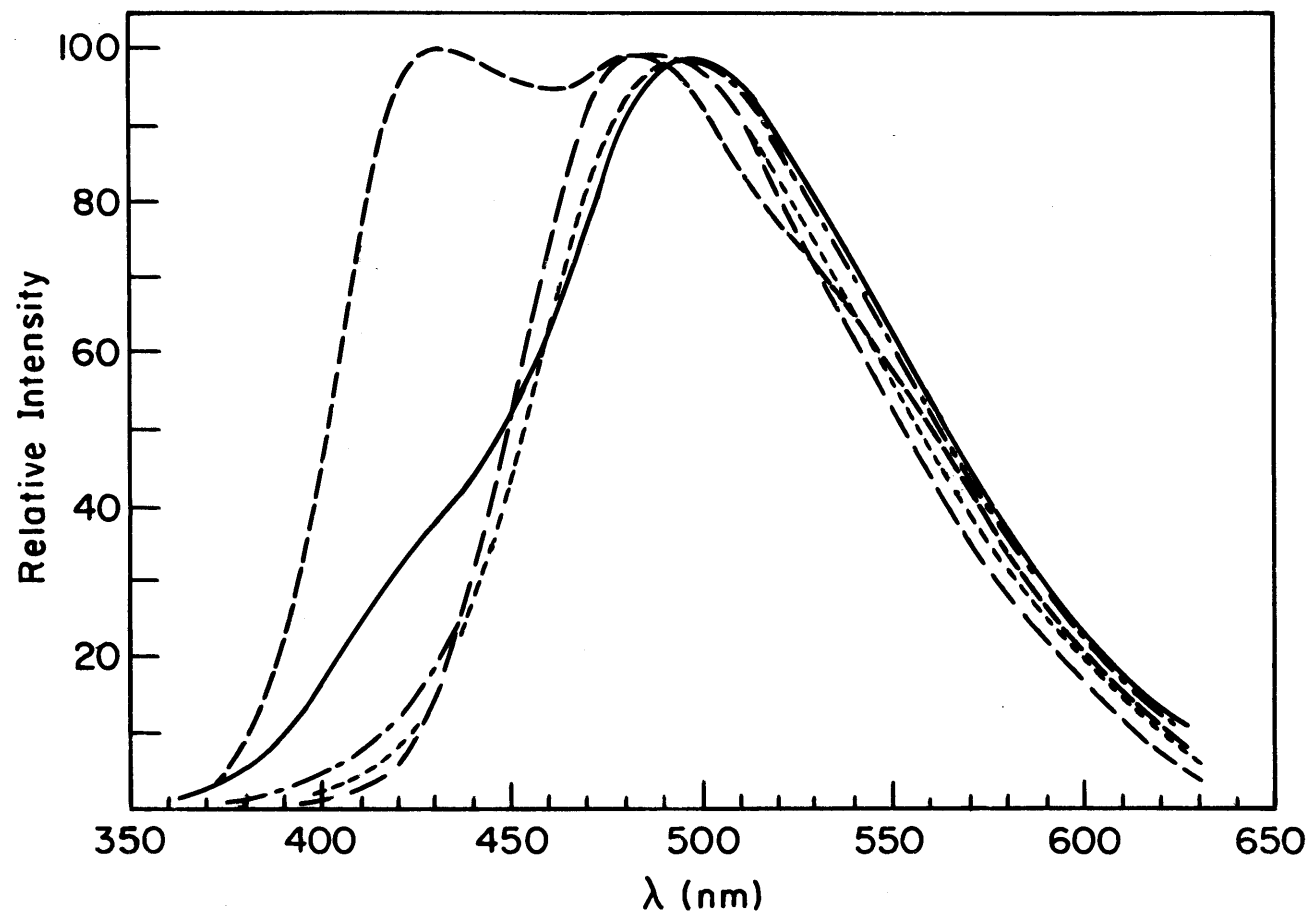


Figure III-9

Normalized Luminol CL Spectra in DMSO-Water
Mixed Solvents 1, 4, 5, 6, and 7

Key

— — — — —	Mixed Solvent 1
- - - - -	Mixed Solvent 4
- - - - -	Mixed Solvent 5
—————	Mixed Solvent 6
- - - - -	Mixed Solvent 7



respectively. These concentrations of reactants produced no CL when mixed solvent 6 was used. However, a spectrum was obtained by increasing the base concentration to 2.5×10^{-2} N KOH and using a 250A slit. Higher concentrations of both NaLUM ($\sim 6 \times 10^{-4}$ M) and base ($\sim 5 \times 10^{-2}$ N) were needed to observe the CL in mixed solvent 7. This spectrum was recorded at the maximum sensitivity of the instrument (x30, 250A slit).

In order to have a basis for comparing the relative intensities of these spectra, each time a reactant concentration was changed to obtain a CL spectrum in a higher mole % water solvent, the CL spectrum for the preceding solvent was also recorded using the new concentrations. Since the spectral distribution of the luminol CL changes only slightly in DMSO and mixed solvents 1-6, the intensity at the maximum of the CL spectrum was taken as a measure of the CL intensity. The intensity of the CL in each of these solvents was then adjusted to the same base concentration, slit width, and sensitivity setting. The calculated relative CL intensities for mixed solvents 1-6 were 10,000, 6440, 1450, 81, 8, and 1, respectively. In DMSO using BTO as the base, the CL intensity is approximately three times the intensity observed in mixed solvent 1. The relative CL data for these solvents are tabulated in Table III-4.

3. Spectral Characteristics of Reaction Product

Absorption ($\lambda_{\text{max}} = 312$ nm), fluorescence ($\lambda_{\text{max}} = 482$, 495 nm), and excitation spectra of the luminol CL reaction products in

Table III-4

Relative Intensities of the Luminol CL
in DMSO and DMSO-Water Mixed Solvents

<u>Solvent</u>			<u>Relative Intensity</u>
DMSO			30,000
Mixed Solvent 1			10,000
"	"	2	6440
"	"	3	1450
"	"	4	81
"	"	5	8
"	"	6	1

DMSO and mixed solvents 1-5 showed that 3-AP⁼ (absorption λ_{max} = 312 nm, fluorescence λ_{max} = 482, 495 nm in these solvents) was one of the products and the yield of 3-AP⁼ was nearly quantitative. Some tailing of the reaction product absorption spectrum was noted in the 360-390 nm region. A study of the basic solvents showed that this absorption could be due to the decomposition of DMSO by the base and oxygen. At the higher water concentrations there also may have been some absorption by unreacted luminol. The reaction product fluorescence spectra in mixed solvents 1-5 are very similar to both the luminol CL and 3-AP⁼ fluorescence spectra in these solvents (see Section III-B-1). The fluorescence spectrum in DMSO sometimes showed a weak band around 400 nm (less than 10 % of main band at 490 nm). This emission probably resulted from either a secondary reaction product (possibly the decarboxylation of 3-AP⁼ to form m-aminobenzoate (fluorescence λ_{max} = 365 nm) or DMSO decomposition by the BT0. This band was not observed in the reaction product fluorescence spectrum in mixed solvent 1.

4. Spectral Characteristics of Substituted Luminol

Absorption and fluorescence data for some methyl derivatives of luminol in DMSO and water are tabulated in Table III-5 for the molecular species and in Table III-6 for the mononegative ions. Extinction coefficients have been calculated for most of these compounds in DMSO. The 1-MeO-3-MeLUM and 4-MeOLUM were isolated in very small amounts and quantitative spectra could not be obtained. Due to the low solubility of all these compounds in water, only qualitative aqueous

Table III-5

Absorption and Fluorescence Characteristics of Some
Methyl Derivatives of Luminol in Water and DMSO

<u>Compound</u>	<u>Water</u> ^a		<u>DMSO</u>	
	Absorption $\lambda_{\max}(\text{nm}), (\epsilon)$	Fluorescence $\lambda_{\max}(\text{nm})$	Absorption $\lambda_{\max}(\text{nm}), (\epsilon)$	Fluorescence $\lambda_{\max}(\text{nm})$
Luminol	294, (5850); 350, (6410)	430	297, (7650); 360, (7720)	410
2-MeLUM	296; 351*	427	301, (6880); 313(s); 360, (8040)	410
3-MeLUM	292; 353*	427	296, (6800); 359, (8170); 370, (8050)	408
2,3- DiMeLUM	292; 357*	None	294, (6870); 364, (8990); 375(s)	None
2-Me-4- MeOLUM	296; 346*	417	299, (7510); 310, (6670); 360, (8160)	410
1-MeO-3- MeLUM	300*; 344	415	301*; 354; 363	406
1-MeOLUM	298, (6800); 342, (5300)	417	300, (~6580); 350, (~4810); 360, (~4760)	405
4-MeOLUM	--- b	--- ^b	297*; 307(s); 358	409

a- HAc-NaAc Buffer (pH = 4.5)

*- Highest intensity band

b- Spectra not recorded

(s)- Shoulder

Table III-6

Absorption and Fluorescence Characteristics of Some Methyl
Derivatives of Luminol in Aqueous Base (2.5×10^{-3} N KOH)
and Basic DMSO (BTO)

<u>Compound</u>	<u>Aqueous Base</u>		<u>DMSO</u>	
	Absorption $\lambda_{\max}(\text{nm}), (\epsilon)$	Fluorescence $\lambda_{\max}(\text{nm})$	Absorption $\lambda_{\max}(\text{nm}), (\epsilon)$	Fluorescence $\lambda_{\max}(\text{nm})$
Luminol	301, (6700); 348, (7520)	410	333, (6370); ^a 370, (6320)	500
2-MeLUM	303; 347*	None	330, (5880); 373, (6750)	525 ^b
3-MeLUM	315; 348*	None	320(s); 340, (6060) 378, (6870)	None
1-MeOLUM	306*; 333; ^c 345(s)	405	300, (~3680); 340(s); 358, (~6240); 372(s)	418
4-MeOLUM	--- d	---d	299; 338; 358*; 374	410; 475 ^e

a- Luminol monoanion

b- Very weak

c- Spectrum recorded using 1.0 N KOH

d- Spectra not recorded

e- Two emissions observed; possible impurity

*- Highest intensity band

spectra were recorded.

The absorption spectra of the molecular species and mononegative ions of 2-MeLUM, 3-MeLUM, and 1-MeOLUM in DMSO are shown in Figures III-10, III-11, and III-12, respectively. The absorption spectra of luminol and 2,3-DiMeLUM in DMSO are compared in Figure III-13. The complete absorption spectra of luminol and 2,3-DiMeLUM in ethanol are shown in Figure III-14. Figure III-15 compares the absorption spectra of luminol, 2-MeLUM, and 2-Me-4-MeOLUM in DMSO.

Qualitatively, the absorption spectrum of 1-MeO-3-MeLUM in DMSO resembles the absorption spectrum of 3-MeLUM although the relative intensities of the bands are reversed and both bands are shifted to longer wavelengths. The absorption spectrum of 4-MeOLUM in DMSO is very similar to the absorption spectra of luminol, 2-MeLUM, and 2-Me-4-MeOLUM with respect to band intensity and absorption maxima. The 4-MeOLUM anion has an absorption spectrum in DMSO which is similar to the spectrum of the 1-MeOLUM anion.

As can be seen from the data in Table III-5, 2-MeLUM, 3-MeLUM, 2,3-DiMeLUM, and 2-Me-4-MeOLUM in aqueous solution show absorption maxima at nearly the same wavelengths as luminol. The shapes of these spectra are also similar to the luminol spectrum. Absorption maxima for 1-MeOLUM and 1-MeO-3-MeLUM in aqueous solution are also similar to luminol. However, for these compounds the short wavelength bands are more intense as was found to be the case for the spectra in DMSO. Absorption spectra of the anions of 2-MeLUM, 3-MeLUM, and 1-MeOLUM in aqueous solution do not differ greatly from

Figure III-10

Absorption Spectra of 2-MeLUM in Neutral and
Basic DMSO

$[C] = 1.0 \times 10^{-4} M$; 1.00 cm cell

Key

—————	Neutral
- - - - -	Basic (BTO)

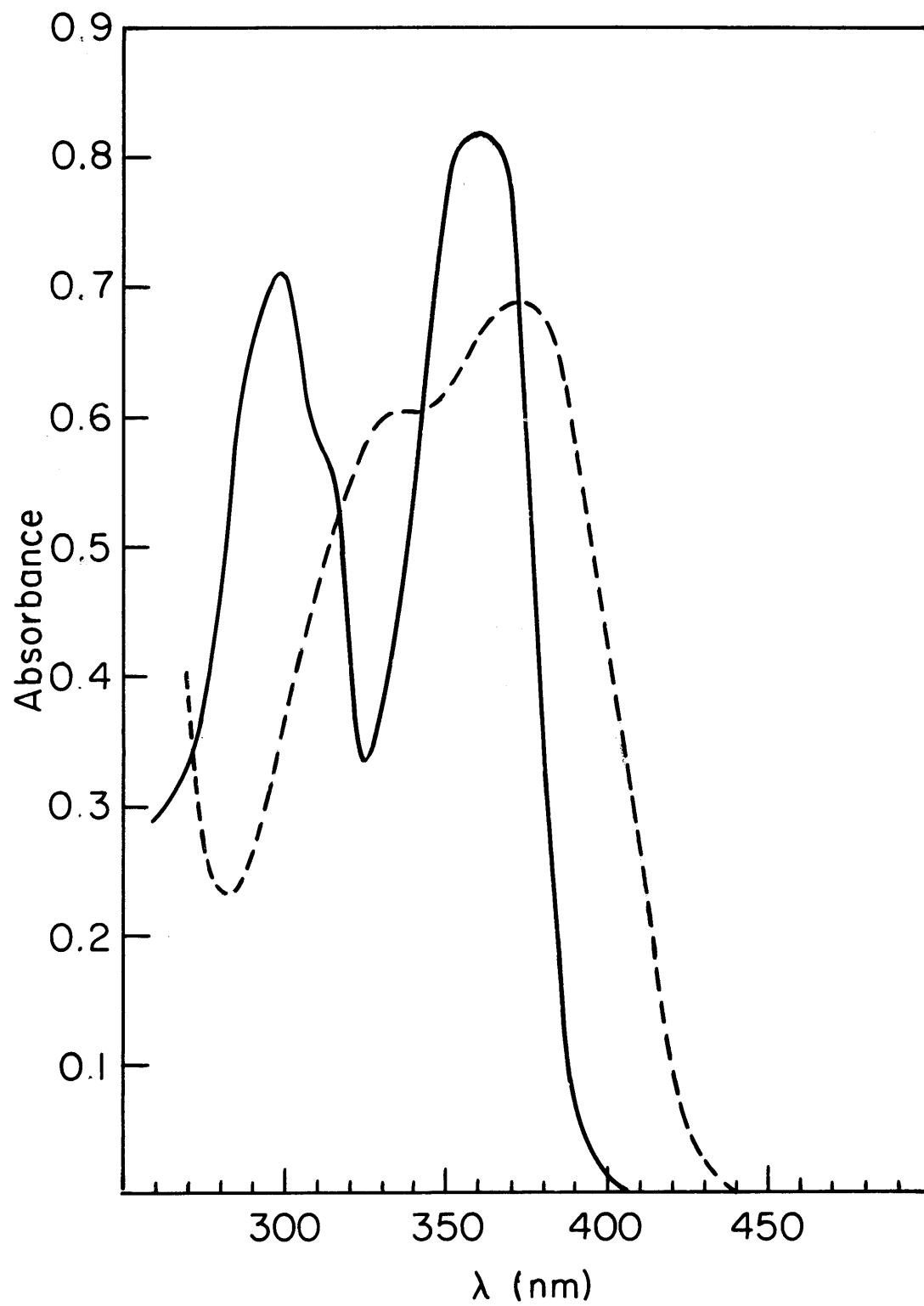


Figure III-11

Absorption Spectra of 3-MeLUM in Neutral and
Basic DMSO

$[C] = 8.7 \times 10^{-5}M$; 1.00 cm cell

Key

—————	Neutral
-----	Basic (BTO)

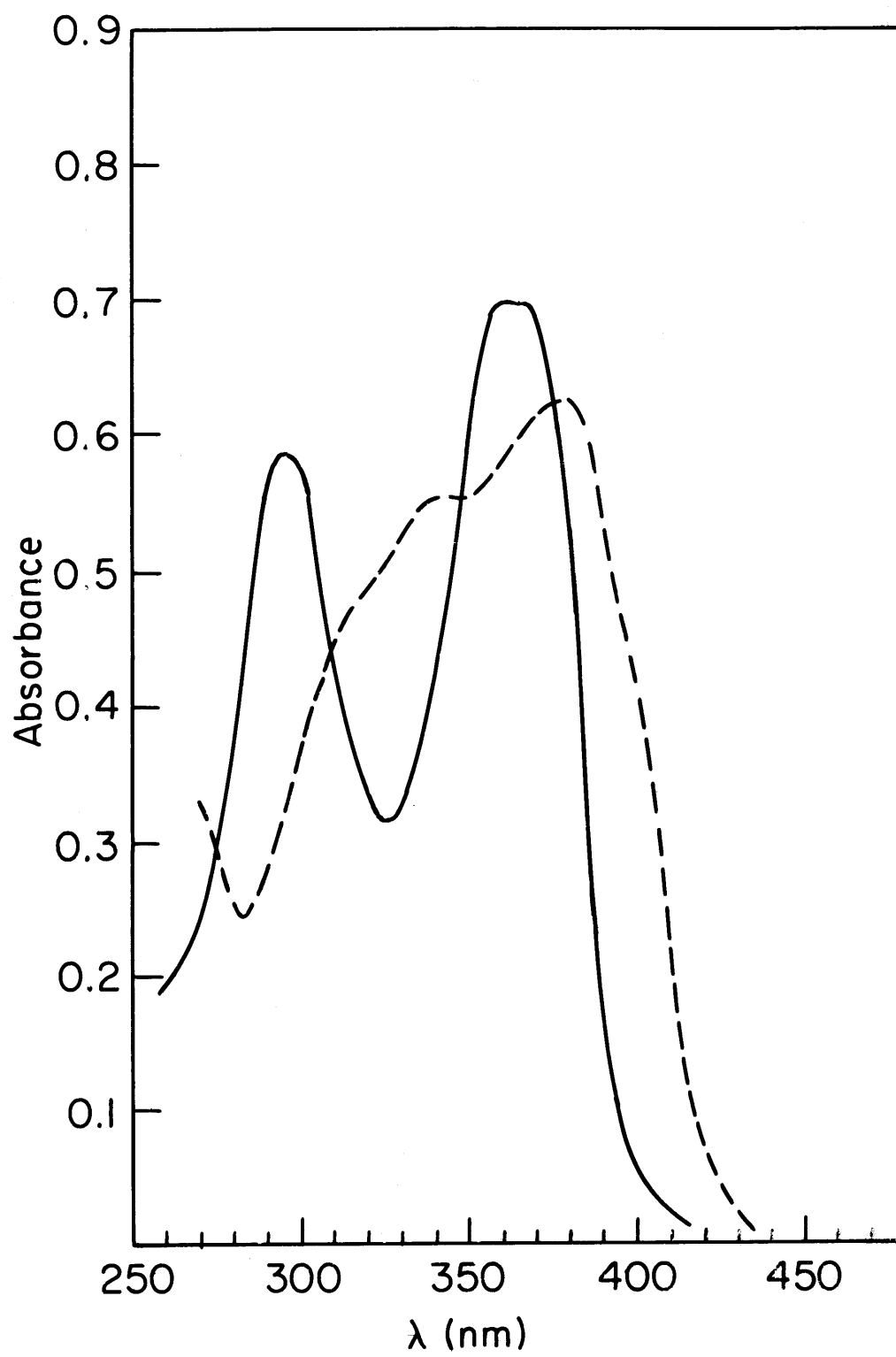


Figure III-12

Absorption Spectra of 1-MeOLUM in Neutral and
Basic DMSO

$[C] = 1.3 \times 10^{-4} M$; 1.00 cm cell

Key

_____	Neutral
-----	Basic (BTO)

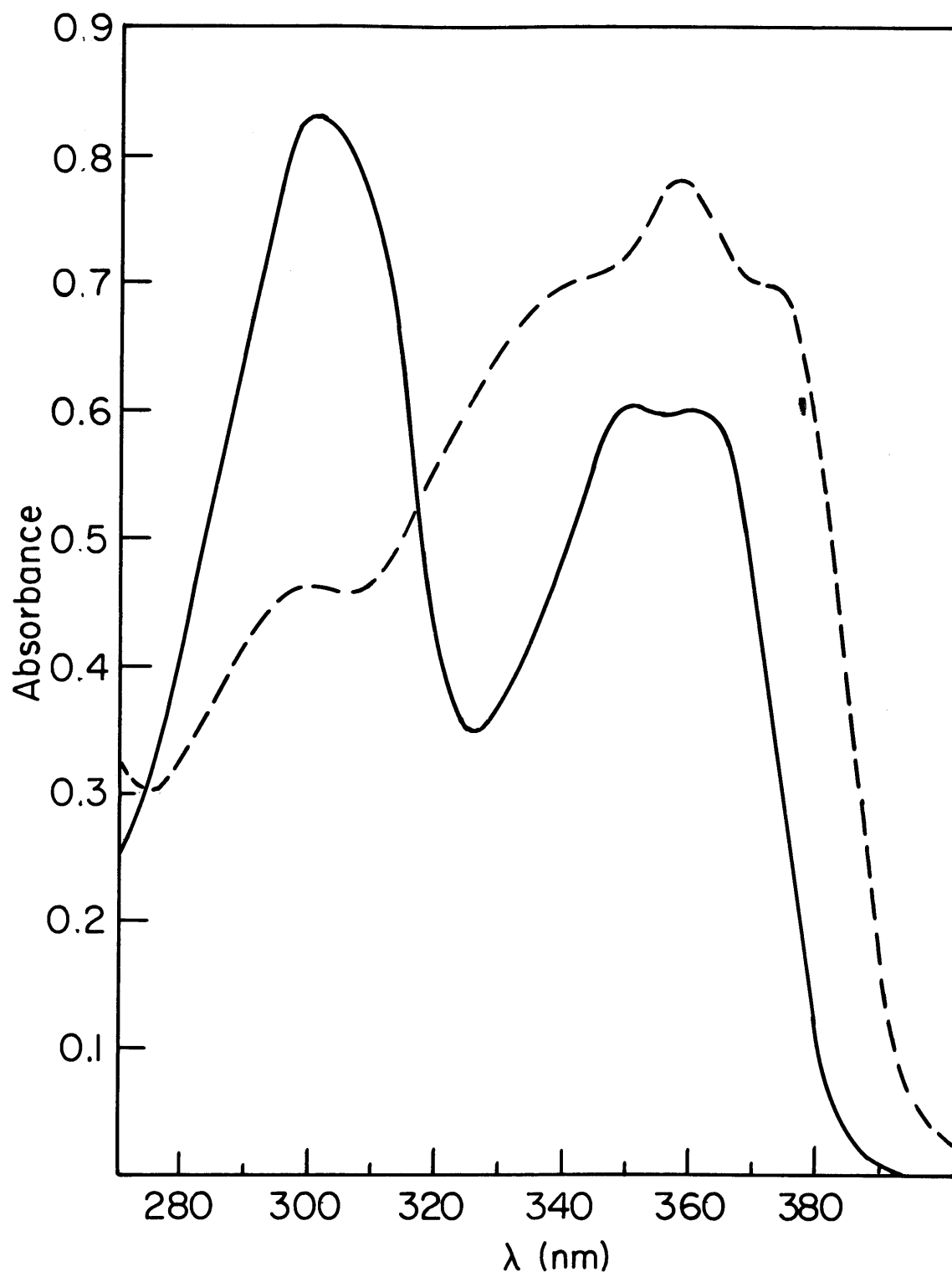


Figure III-13

Absorption Spectra of Luminol, 2,3-DiMeLUM,
PD, and 2,3-DiMePD in DMSO

Key

— — — — —	Luminol
—————	2,3-DiMeLUM
- - - - -	PD
— — — — —	2,3-DiMePD

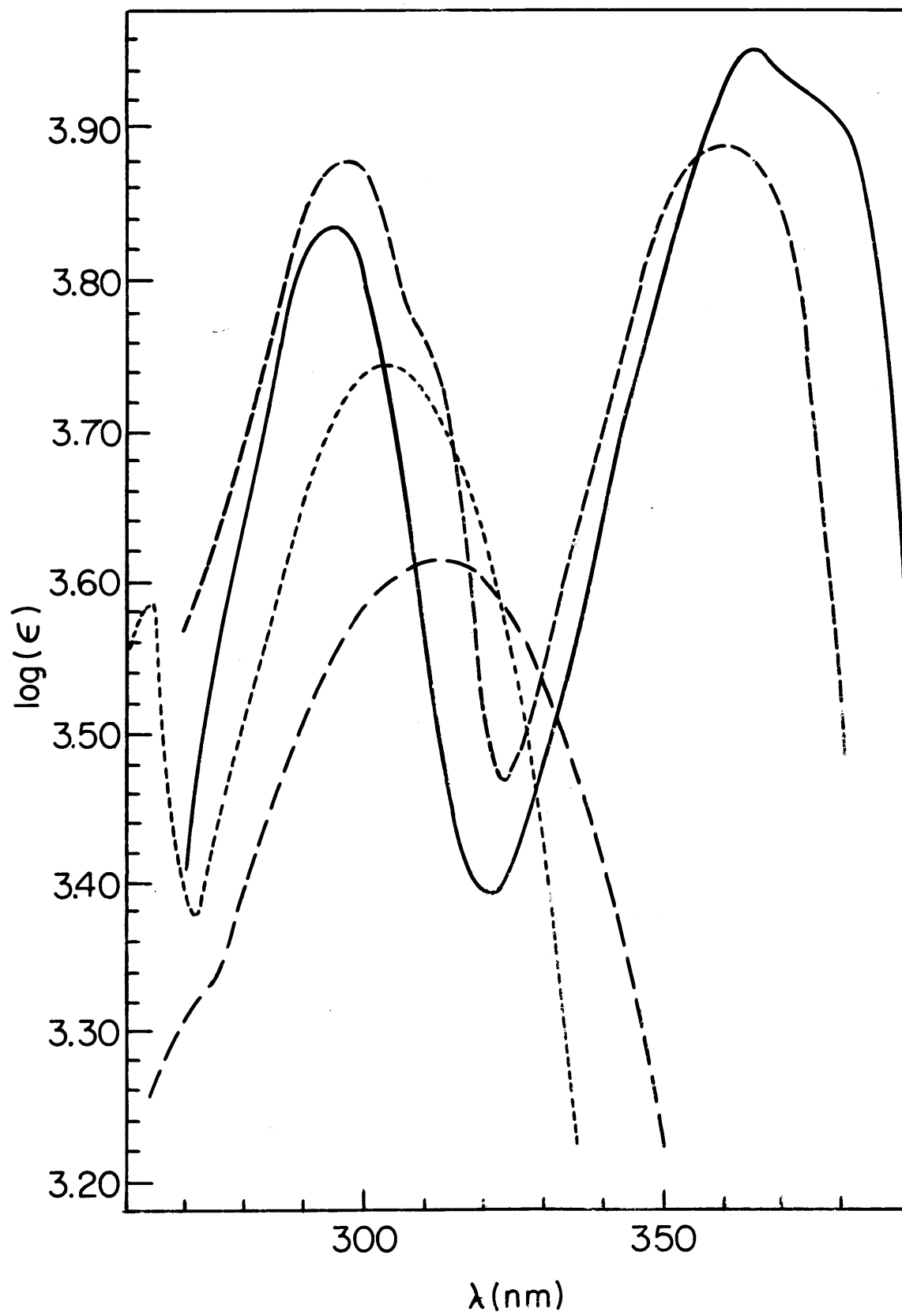


Figure III-14

Absorption Spectra of Luminol, 2,3-DiMeLUM, PD,
2,3-DiMePD, and 6-DiethylaminoPD in Ethanol

Key

— — — — —	Luminol
- - - - -	2,3-DiMeLUM
—————	PD
- - - - -	2,3-DiMePD
- - - - -	6-DiethylaminoPD

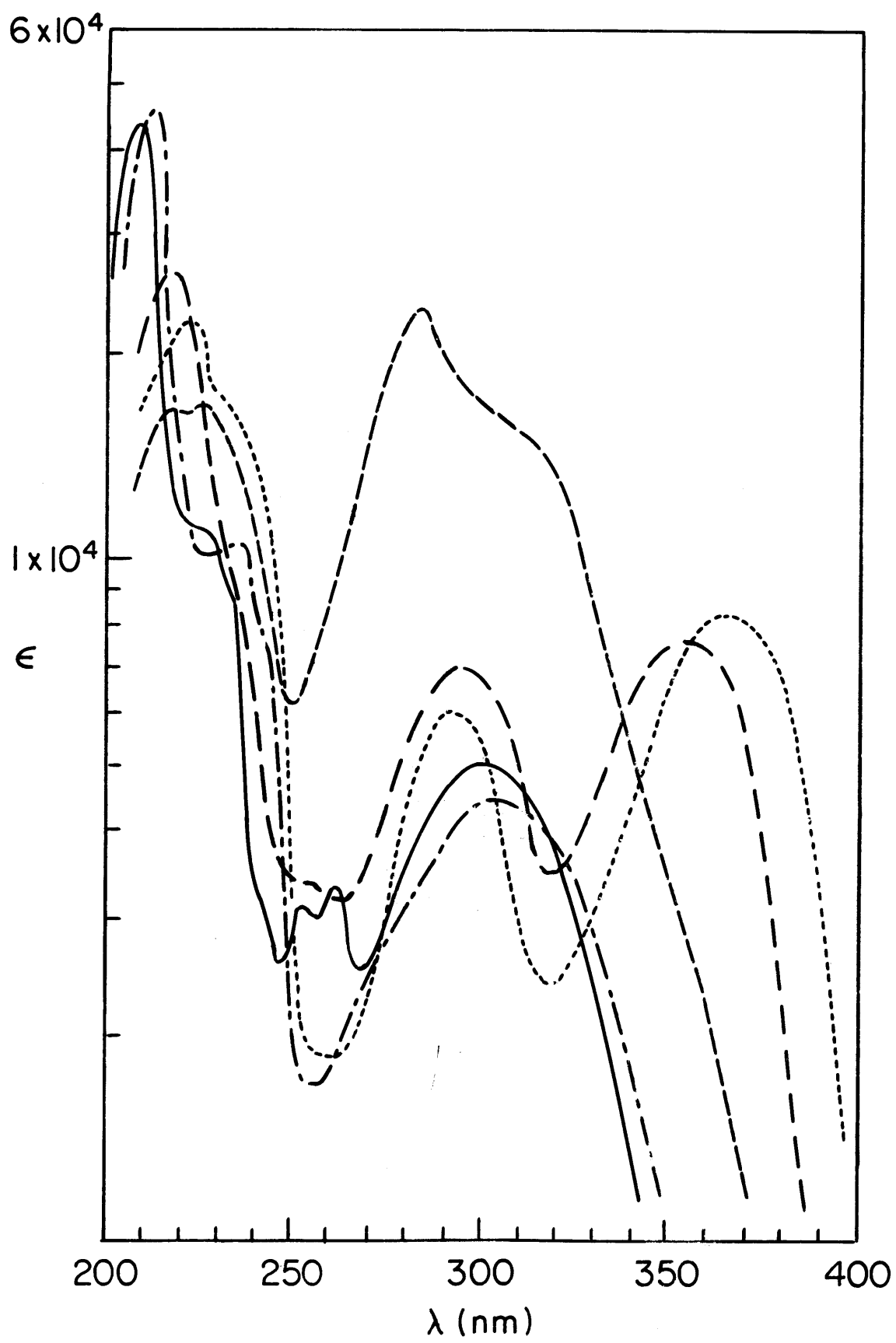
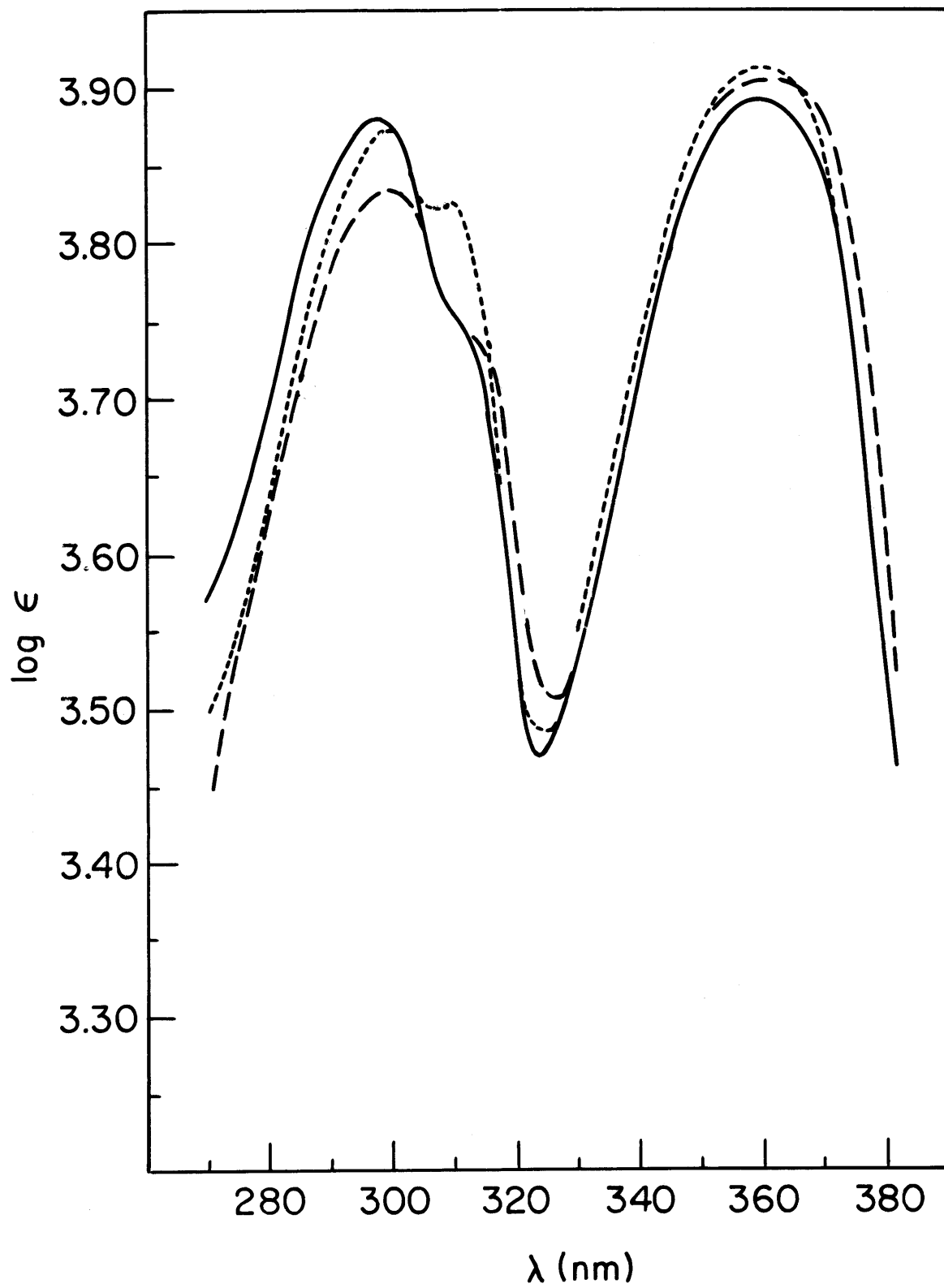


Figure III-15

Absorption Spectra of Luminol, 2-MeLUM, and
2-Me-4-MeOLUM in DMSO

Key

—————	Luminol
- - - - -	2-MeLUM
- - - - -	2-Me-4-MeOLUM



the aqueous luminol anion spectrum. It should be noted that a base concentration of 2.5×10^{-3} N KOH was not sufficient to remove the proton from 1-MeOLUM. This absorption spectrum was recorded using 1.0 N KOH.

Solutions of the methyl-substituted luminols in DMSO were checked to see if any CL could be observed when oxygen and base were added. The mono-methyl derivatives (2-Me-, 3-Me-, 1-MeO-, and 4-MeOLUM) did show a very faint, short-lived CL upon the addition of a large excess of BTO. Since absorption spectra of the anions of these compounds were obtained in DMSO with a small amount of BTO added and these solutions appeared to be stable, the origin of the CL is questionable. The weak emission could have resulted from small traces of luminol in these compounds and further study was not warranted.

5. Spectral Characteristics of PD and Substituted PD

Absorption and fluorescence data for PD, MePD, and 2,3-DiMePD in water and DMSO are tabulated in Table III-7 for the molecular species. Data for 6-diethylaminoPD (29) in DMSO are also included. The spectral characteristics of the PD and MePD monoanions in water and DMSO and the PD dianion in DMSO are shown in Table III-8. The dianion was not formed in aqueous solution at the base concentrations studied (up to 1.0 N KOH).

As can be seen in Table III-7, methyl substitution shifts the absorption spectra of PD in water ($\lambda_{\text{max}} = 292 \text{ nm}$) and in DMSO

Table III-7

Absorption and Fluorescence Characteristics of PD
and Substituted PD in Water and DMSO

<u>Compound</u>	<u>Water^a</u>		<u>DMSO</u>	
	Absorption $\lambda_{\max}(\text{nm}), (\epsilon)$	Fluorescence $\lambda_{\max}(\text{nm})$	Absorption $\lambda_{\max}(\text{nm}), (\epsilon)$	Fluorescence $\lambda_{\max}(\text{nm})$
PD	292	None	305, (5540)	435
MePD	298	None	309	430
2,3-DiMePD	299	None	311, (4130)	None
6-Diethyl-aminoPD	---b	---b	289, (24,550)	410

a- HAc-NaAc buffer (pH = 4.5)

b- Spectra not recorded

Table III-8

Absorption and Fluorescence Characteristics of PD
and MePD in Aqueous Base and Basic DMSO

<u>Compound</u>	<u>Aqueous Base^a</u>		<u>DMSO^b</u>	
	Absorption $\lambda_{\max}(\text{nm}), (\epsilon)$	Fluorescence $\lambda_{\max}(\text{nm})$	Absorption $\lambda_{\max}(\text{nm}), (\epsilon)$	Fluorescence $\lambda_{\max}(\text{nm})$
PD Monoanion	310, (5500)	485	364, (3640)	550
PD Dianion	--- ^c	--- ^c	330, (2900); ^d 402, (4400)	625 ^e
MePD Monoanion	313	485	367	545

a- 2.5×10^{-3} N KOH

b- DMSO + BTO

c- Dianion not observed in water

d- High ϵ because of solvent absorption

e- Very weak

($\lambda_{\max} = 305$ nm) to larger wavelengths ($\lambda_{\max} = 298, 309$ nm for MePD and $\lambda_{\max} = 299, 311$ nm for 2,3-DiMePD in water and DMSO, respectively). Aqueous solutions of these compounds were not fluorescent. PD and MePD fluoresce weakly in DMSO ($\lambda_{\max} = 435, 430$ nm, respectively).

The absorption spectra of PD and 2,3-DiMePD in DMSO are shown in Figure III-13 along with the spectra of luminol and 2,3-DiMeLUM in DMSO. The complete absorption spectra of PD ($\lambda_{\max} = 301$ nm), 2,3-DiMePD ($\lambda_{\max} = 303$ nm), and 6-diethylaminoPD ($\lambda_{\max} = 284$ nm) in ethanol were also recorded and are shown in Figure III-14 where they are compared to the spectra of luminol and 2,3-DiMeLUM in ethanol.

Removal of a proton from PD (see Table III-8) results in a very large shift in the absorption spectrum (305 nm \rightarrow 364 nm) in DMSO compared to the shift observed in water (292 nm \rightarrow 310 nm). Similar shifts are observed for the MePD monoanion. Absorption spectra of NaPD in various DMSO-water mixed solvents showed that the absorption maximum shifts to shorter wavelengths as the amount of water in the mixed solvent increases. The shape and intensity of this band change very little and there are no isosbestic points. The absorption data for DMSO, water, and mixed solvents 6, 10, and 12 are tabulated in Table III-9.

DMSO solutions of the monoanions of PD and MePD show a weak yellow fluorescence ($\lambda_{\max} = 550$ nm and 545 nm, respectively) and the excitation spectra are similar to the absorption spectra of the monoanions.

Table III-9

Effect of the Mixed Solvent Composition on the
Absorption Maximum of NaPD

<u>Solvent</u>	<u>λ_{\max}(nm)</u>
DMSO	364
Mixed Solvent 6	352
" " 10	327
" " 12	315
Water (2.5×10^{-3} <u>N</u> KOH)	310

The absorption spectrum of the PD dianion ($\lambda_{\text{max}} = 330 \text{ nm}, 402 \text{ nm}$) in DMSO is shown in Figure III-16 along with the absorption spectra of PD and the PD monoanion in DMSO. The dianion spectrum was recorded using a carefully vacuum-degassed DMSO solution of NaPD containing an excess of BTO. The procedure for preparing this solution was the same as that used to obtain the luminol dianion spectrum. Since the BTO resulted in some decomposition of the DMSO, the dianion absorption in the region 270 nm to 350 nm is distorted by solvent absorption. It is particularly interesting to note the similarity between this spectrum and the absorption spectrum of the luminol dianion ($\lambda_{\text{max}} = 310 \text{ nm}, 393 \text{ nm}$) shown in Figure III-2. The fluorescence of the PD dianion is very weak ($\lambda_{\text{max}} = 625 \text{ nm}$).

Solutions of NaPD in DMSO exhibit a weak yellow CL when exposed to oxygen and an excess of BTO.

6. Infrared Spectra

An attempt was made to study the keto-enol tautomeric equilibrium of luminol, PD and the mono-sodium salts of these compounds using infrared spectroscopy. However, spectra in the standard infrared solvents could not be obtained because of the low solubility of the compounds in these solvents. KBr spectra of these compounds were recorded and all showed the presence of the carbonyl group (PD, 1660 cm^{-1} ; NaPD, 1640 cm^{-1} ; LUM, 1650 cm^{-1} ; NaLUM, 1640 cm^{-1}). Absorption characteristic of the hydroxyl group was not observed for any of the compounds.

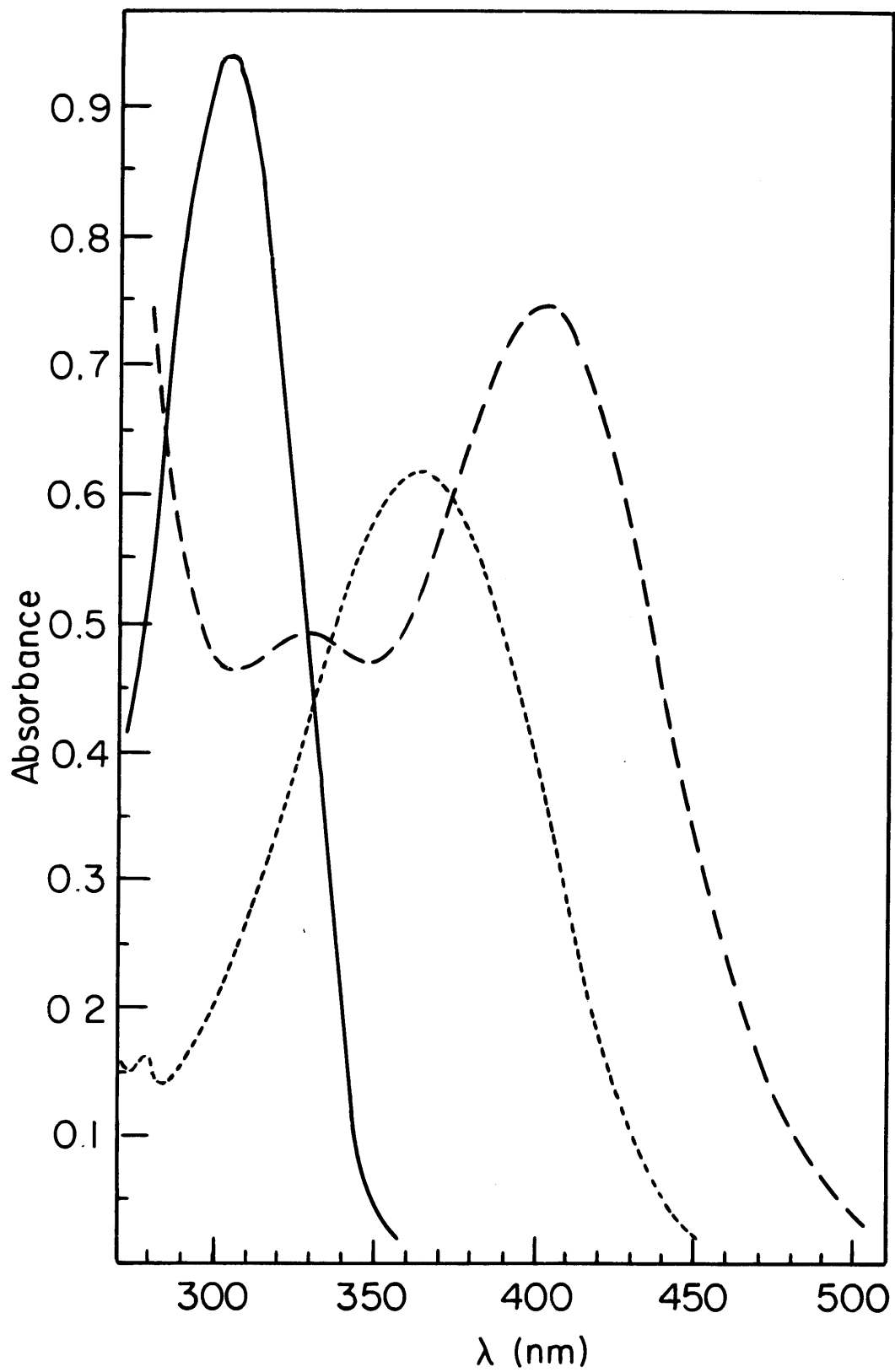
Figure III-16

Absorption Spectra of PD, PD Monoanion, and
PD Dianion in DMSO

$[C] = 1.7 \times 10^{-4} M$; 1.00 cm cell

Key

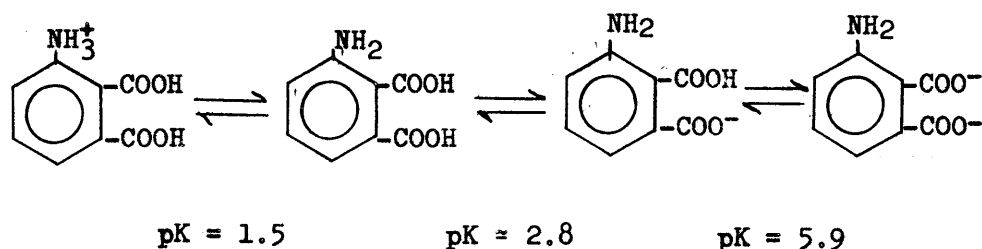
—————	PD
- - - - -	PD Monoanion
- - - - -	PD Dianion



B. 3-Aminophthalic Acid Study

1. Absorption and Fluorescence Characteristics

Absorption and fluorescence spectra of 3-APA in aqueous solution were recorded as a function of pH. Changes in the absorption spectra as well as comparisons with analogous compounds were used to determine the pK_a 's for the equilibria shown below.



For pH's greater than about 12.5 a decrease in fluorescence intensity was observed with no apparent change in the absorption spectrum. It was not determined whether this was due to an excited-state equilibrium or to the decomposition of $3-AP^=$ at high base concentrations. The latter is a strong possibility since solutions of $3-AP^=$ in 10^{-3} N KOH decomposed after a few days.

The major part of this investigation was concerned with the spectral characteristics of $3-AP^=$ since this has been identified as the emitting species in the luminol chemiluminescent reaction. The main absorption band of $3-AP^=$ occurs at 303 nm ($\epsilon = 2430$) in aqueous solution and at 312 nm ($\epsilon = 2750$) in DMSO. For mixed solvents 1-8 the absorption maximum remained at 312 nm with no observable change

in extinction coefficient. Higher concentrations of water cause the band to shift to the aqueous spectrum.

Figure III-17 shows the fluorescence spectra of $3\text{-AP}^=$ in water ($\lambda_{\text{max}} = 425 \text{ nm}$) and DMSO ($\lambda_{\text{max}} = 482, 495 \text{ nm}$; referred to as the 490 nm band). The fluorescence spectra in mixed solvents 1-3 are similar to the DMSO spectrum except that the intensities of the 482 nm and 495 nm peaks are reversed. The fluorescence spectra obtained using mixed solvents 4-9 and their relative intensities are shown in Figure III-18. It should be noted that the short wavelength band ($\sim 410 \text{ nm}$) is blue-shifted relative to the aqueous spectrum. Excitation spectra are the same for both bands and correspond to the $3\text{-AP}^=$ absorption spectrum in DMSO.

In order to determine if the two bands observed in the $3\text{-AP}^=$ fluorescence spectra in the mixed solvents were dependent upon the base concentration, a solution of $3\text{-AP}^=$ in basic mixed solvent 7 ($2.5 \times 10^{-3} \text{ N KOH}$) was titrated with a solution of HCl in mixed solvent 7. Absorption and fluorescence spectra were recorded after each addition of HCl and showed that the intensities of both bands in the $3\text{-AP}^=$ fluorescence spectrum in mixed solvent 7 did not change until enough acid was added to form the monoanion.

The $3\text{-AP}^=$ absorption spectrum ($\lambda_{\text{max}} = 312 \text{ nm}$) and fluorescence spectrum ($\lambda_{\text{max}} = 495 \text{ nm}$) in DMF are similar to the absorption and fluorescence spectra in DMSO. The fluorescence spectra in DMF-water mixed solvents also showed the development of a new band at about 410 nm. However, for the same mixed solvent composition the

Figure III-17

Fluorescence Spectra of 3-AP⁺ in Water, DMSO,
and Mixed Solvents 1-3

Key

————— Water (2.5×10^{-3} N KOH)

----- DMSO (BTO)
Mixed Solvents 1-3
(2.5×10^{-3} N KOH)

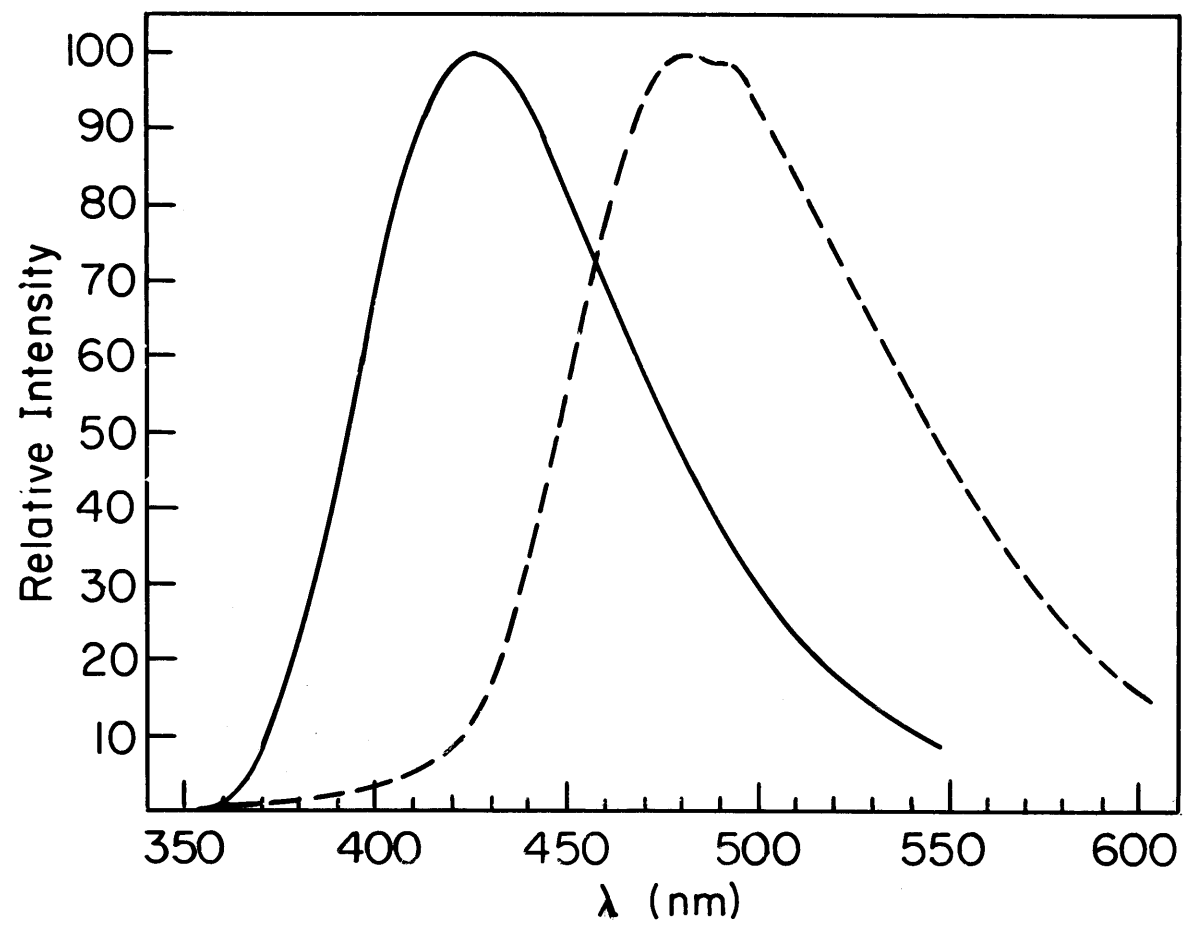


Figure III-18

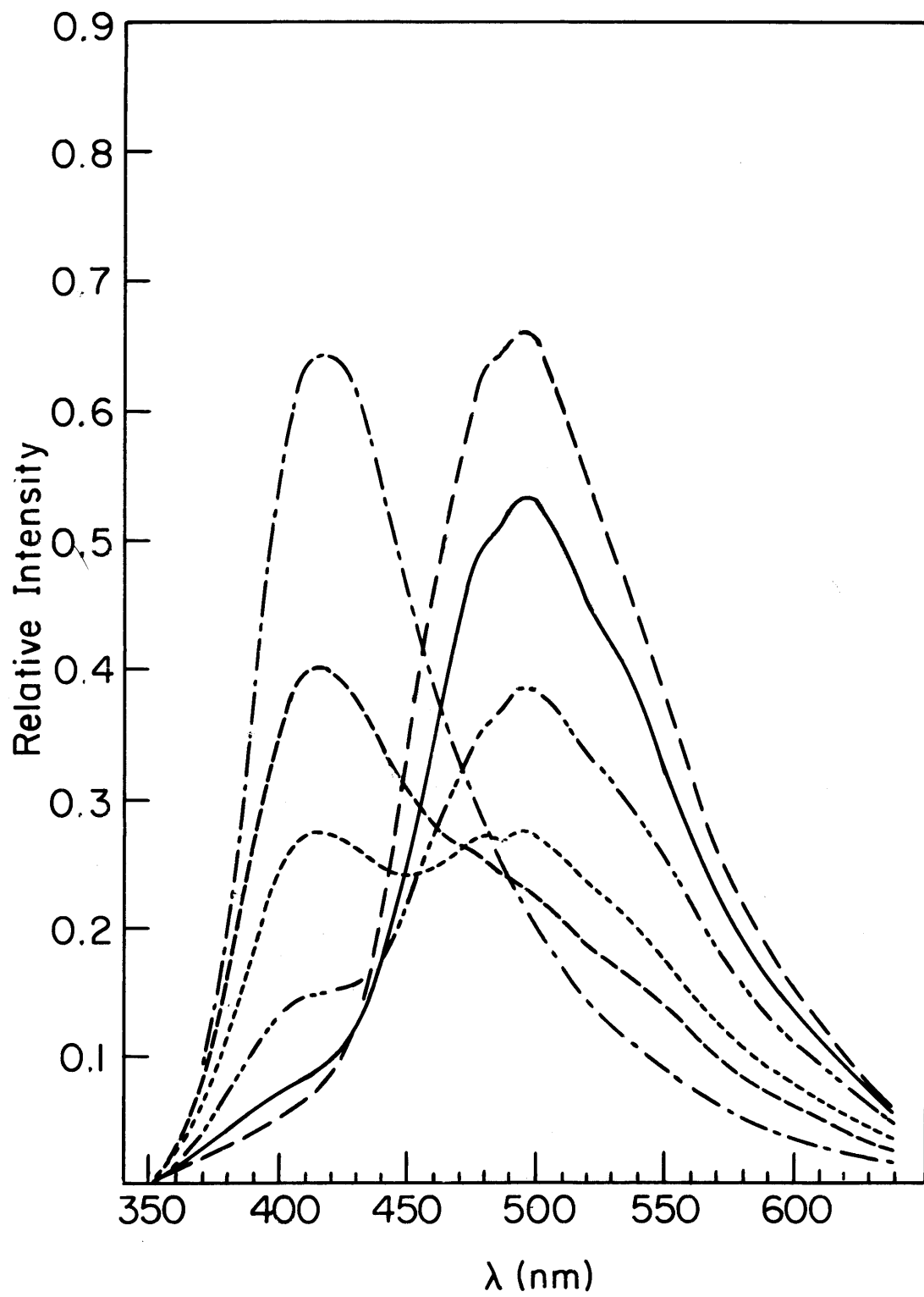
Fluorescence Spectra of 3-AP⁻ in Mixed Solvents 4-9

[C] $\approx 5 \times 10^{-5}M$ for all spectra

Key

— — — — —	Mixed Solvent 4		
—————	"	"	5
- - - - -	"	"	6
- - - - -	"	"	7
— — — — —	"	"	8
- - - - -	"	"	9

All Mixed Solvents - $2.5 \times 10^{-3}N$ KOH



relative intensity of the short wavelength band to the long wavelength band was greater in the DMF-water mixed solvent than in the DMSO-water mixed solvent. For example, the fluorescence spectrum in the DMSO-water mixed solvent 7 has two bands of about equal intensity. For a DMF-water mixed solvent of similar composition the 495 nm band has practically disappeared.

2. Fluorescence Quantum Yields

The fluorescence quantum yields for 3-AP[±] in water, DMSO, and various DMSO-water mixed solvents were determined and are tabulated in Table III-10. In water and DMSO the ϕ_f 's are about 25% and 11%, respectively. The plot of ϕ_f versus mole % water in Figure III-19 shows that ϕ_f begins to decrease after about 10 mole % water and reaches a minimum for mixed solvent 7 (41 mole % water). This is the mixed solvent in which the 3-AP[±] fluorescence shows two bands (410 nm, 490 nm) of about equal intensity.

3. Analogous Compounds

The spectral characteristics of some analogous compounds are given in Table III-11. The o-aminobenzoate is the only compound which shows the same behavior as 3-AP[±] in water ($\lambda_{\max} = 400$ nm), in DMSO ($\lambda_{\max} = 475$ nm), and in the mixed solvents ($\lambda_{\max} = 380, 475$ nm). The fluorescence quantum yields for o-dimethylaminobenzoate and p-amino-benzoate in DMSO and p-dimethylaminobenzoate in water are very low and fluorescence spectra could not be obtained for these compounds.

Table III-10

3-AP⁺ Fluorescence Quantum Yields in DMSO, Water,
and DMSO-Water Mixed Solvents

Solvent	ϕ_f (%)
DMSO ^a	11.0
Water ^b	25.2
Mixed Solvent 3 ^b	12.0
" " 4	12.0
" " 5	10.3
" " 6	8.7
" " 7	8.0
" " 8	8.3
" " 9	11.7

a- DMSO + BTO

b- $2.5 \times 10^{-3} \text{N}$ KOH

Figure III-19

The Fluorescence Quantum Yield of 3-AP⁻ as a Function
of the Mixed Solvent Composition

ϕ_f versus Mole % Water

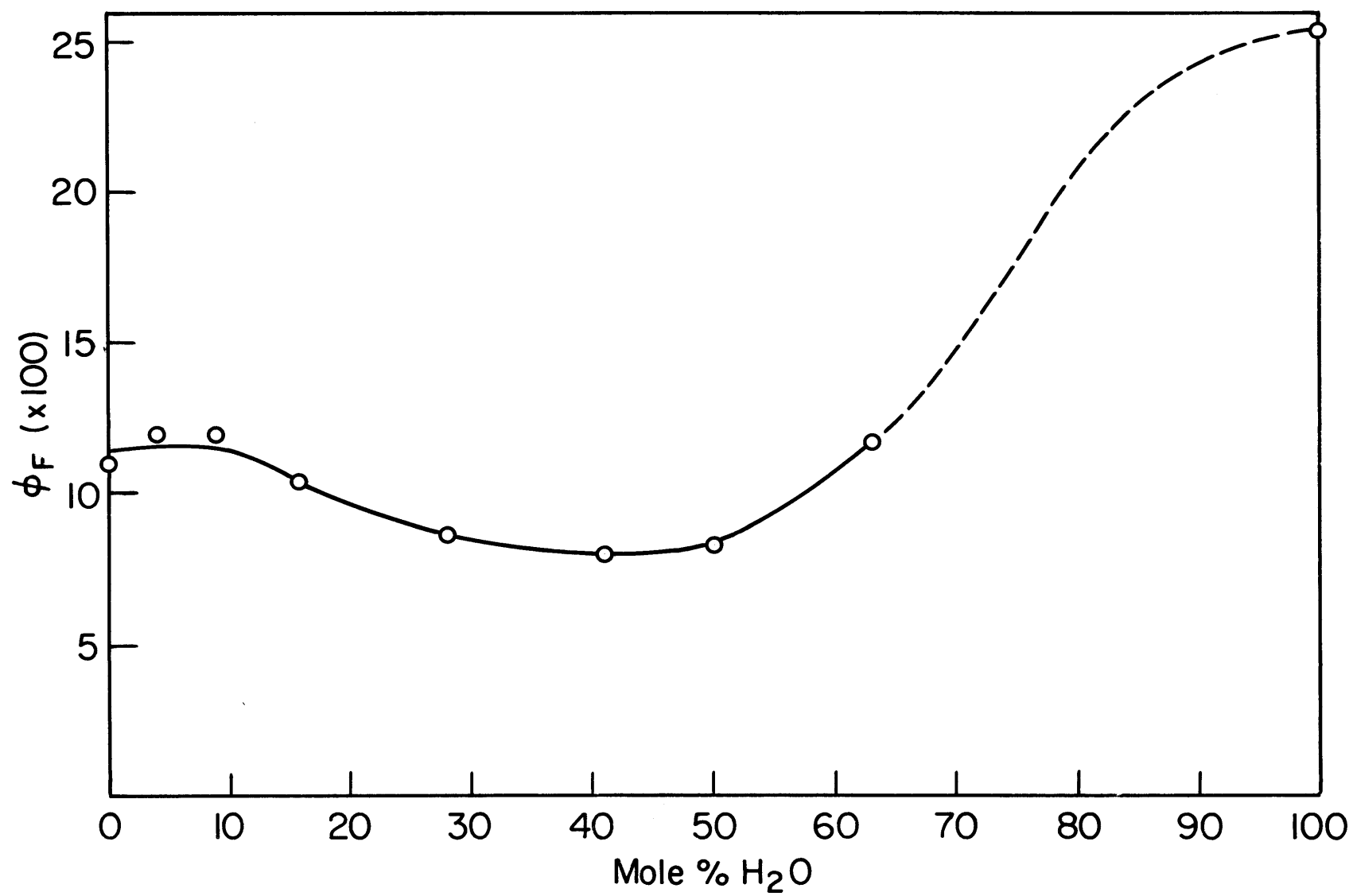


Table III-11

Absorption and Fluorescence Characteristics of Analogous Compounds
in Aqueous Base and Basic DMSO

<u>Compound</u>	<u>Aqueous Base^a</u>		<u>DMSO^b</u>	
	Absorption λ_{\max} (nm)	Fluorescence λ_{\max} (nm)	Absorption λ_{\max} (nm)	Fluorescence λ_{\max} (nm)
3-Amino-phthalate	303	425	312	490 (\sim 410) ^c
4-Amino-phthalate	260	406	280	368
o-Amino-benzoate	310	400	317	475 (\sim 380) ^c
o-Dimethyl-aminobenzoate	260, 300(s)	420	268, 300(s)	---- ^d
m-Amino-benzoate	300	407	306	365
m-Dimethyl-aminobenzoate	306	437	310	375
p-Amino-benzoate	290	334	336	---- ^d
p-Dimethyl-aminobenzoate	289	---- ^d	275	350

a- $2.5 \times 10^{-3} \text{N}$ KOH

(s)- Shoulder

b- DMSO + BTO

c- Short wavelength band in mixed solvents

d- ϕ_f too small to obtain spectrum

C. Stopped-Flow Experiments

1. CL Decay in DMSO

In the stopped-flow experiments the concentrations of luminol, oxygen, and BTO were varied independently and the CL decay curves were recorded. A typical decay curve is shown in Figure III-20. When the CL intensity reached a steady-state value, the flow was stopped ($t=0$) and the decay of the light was monitored. Depending on the concentrations of reactants, the chemiluminescent reaction was essentially over in 5-15 seconds. All the reactant concentrations given below are the values before mixing.

The effect of various luminol concentrations on the nature of the CL decay curves was studied by varying the concentration of NaLUM (NaLUM was used for all the stopped-flow experiments) from 1.8×10^{-4} M to 4.5×10^{-5} M while the BTO concentration was held constant at 1.8×10^{-3} M and the NaLUM solutions were saturated with oxygen (2×10^{-3} M). Several decay curves were run for each NaLUM concentration, and these curves were very reproducible. The higher NaLUM concentrations showed CL decay curves similar to that shown in Figure III-20. Lowering the concentration of NaLUM, not only results in a decrease in the CL intensity, but also causes the maximum in the CL decay curve to disappear. The CL decay curve for the 4.5×10^{-5} M luminol solution is shown in Figure III-21. The different amplifier feedback resistors, R_{FB} , used to record the curves in Figure III-20 (10K Ω) and Figure III-21 (51K Ω) should be noted. Changing R_{FB} from

Figure III-20

Typical Stopped-Flow CL Decay Curve

Key

Before Mixing:

$$[\text{NaLUM}] = 1.0 \times 10^{-4} \text{M}$$

$$[\text{BTO}] = 2.3 \times 10^{-3} \text{M}$$

$$[\text{O}_2] = 2 \times 10^{-3} \text{M}$$

$$R_{\text{FB}} = 10\text{K}$$

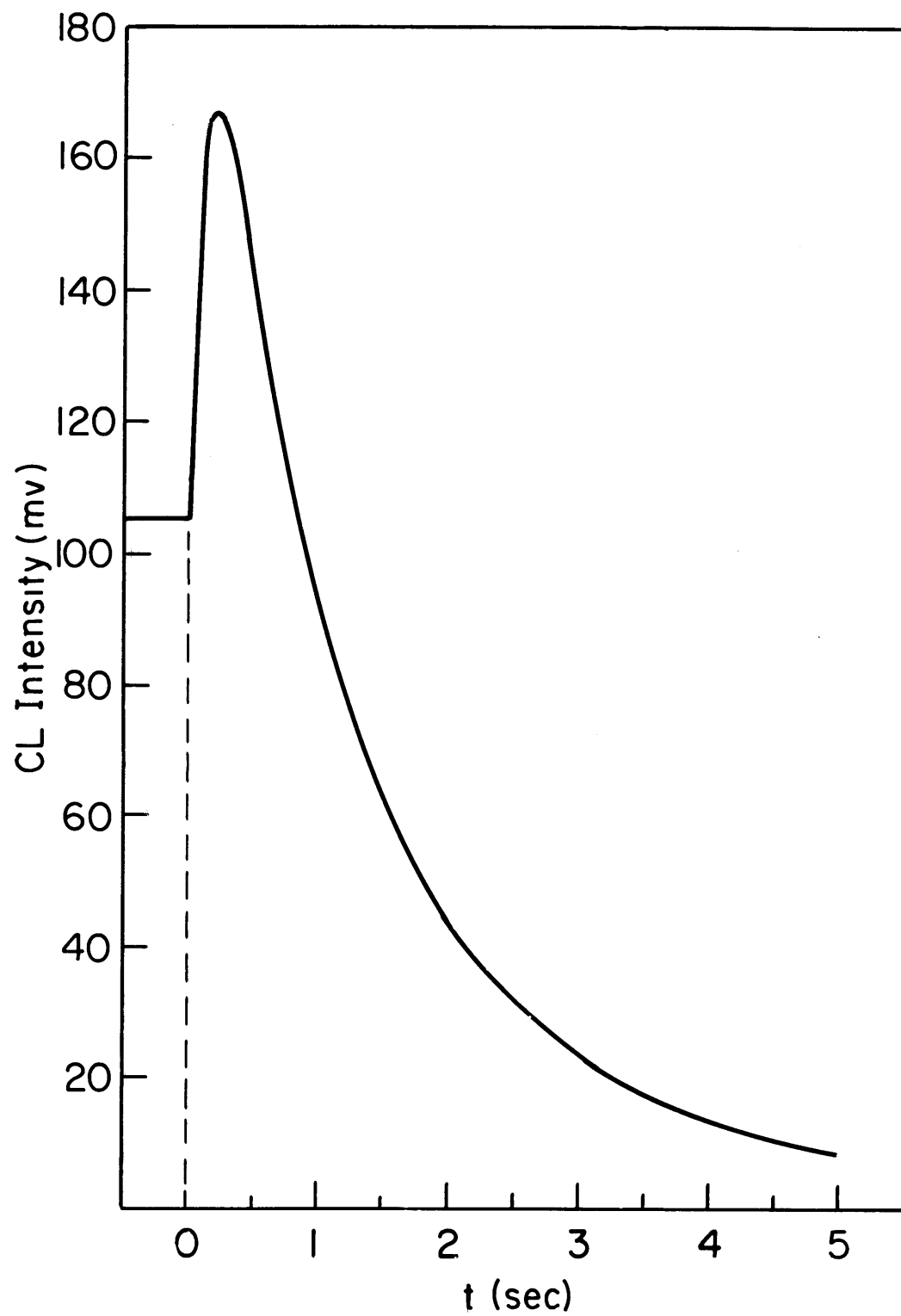


Figure III-21

CL Decay Curve for Low Luminol Concentration

Key

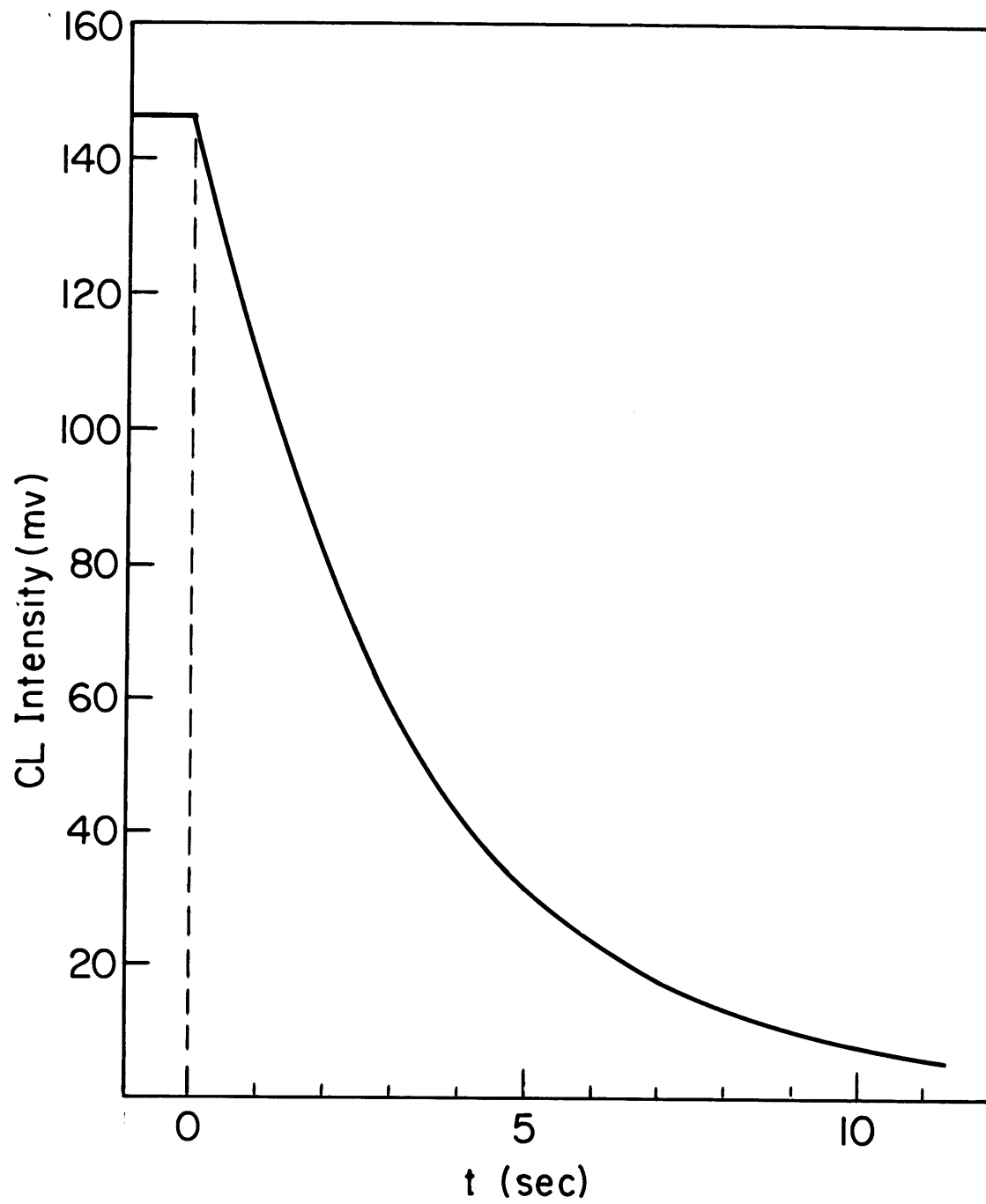
Before Mixing:

$$[\text{NaLUM}] = 4.5 \times 10^{-5}\text{M}$$

$$[\text{BTO}] = 1.8 \times 10^{-3}\text{M}$$

$$[\text{O}_2] = 2 \times 10^{-3}\text{M}$$

$$R_{\text{FB}} = 51\text{K}$$



10K Ω to 51K Ω increases the amplifier's sensitivity by a factor of 5.1.

Figure III-22 shows a plot of Log (I) versus t for the decay curve shown in Figure III-21. As can be seen, the plot is linear and indicates that the reaction has become pseudo-first order in luminol. The pseudo-first order rate constant, K' , is $1.4 \times 10^{-1} \text{ sec}^{-1}$. Another set of experiments using new solutions ($[\text{BTO}] = 2.3 \times 10^{-3} \text{ M}$; $[\text{O}_2] = 2 \times 10^{-3} \text{ M}$) showed that for a NaLUM concentration of $2.0 \times 10^{-5} \text{ M}$ the reaction had become pseudo-first order and the rate constant was $2.0 \times 10^{-1} \text{ sec}^{-1}$.

The experiments using various concentrations of BTO while the luminol and oxygen concentrations remained constant were difficult to run because of the unstable nature of the DMSO-BTO solutions. This is especially true for the low BTO concentrations. However, when freshly prepared BTO solutions were deoxygenated by bubbling with DMSO presaturated nitrogen, they were sufficiently stable and reproducible CL decay curves were obtained. The NaLUM and oxygen concentrations were held constant at $1.0 \times 10^{-3} \text{ M}$ and $2 \times 10^{-3} \text{ M}$, respectively, while the BTO concentration was varied from $6.8 \times 10^{-4} \text{ M}$ to $4.6 \times 10^{-5} \text{ M}$. In general, the decay curves were similar in appearance to the curves observed in the luminol study above. The normalized decay curves for the two extreme concentrations of BTO are shown in Figure III-23. Over this range of BTO concentrations the CL intensity (measured at the maximum) showed some very large changes. For example, a 225 mv signal was recorded for the $6.8 \times 10^{-4} \text{ M}$ BTO solution ($R_{\text{FB}} = 10\text{K}\Omega$) while the signal for the $4.6 \times 10^{-5} \text{ M}$ solution was only 0.75 mv

Figure III-22

Plot of Log (I) Versus Time for the CL Decay

Curve Shown in Figure III-21

$$k' = 1.4 \times 10^{-1} \text{sec}^{-1}$$

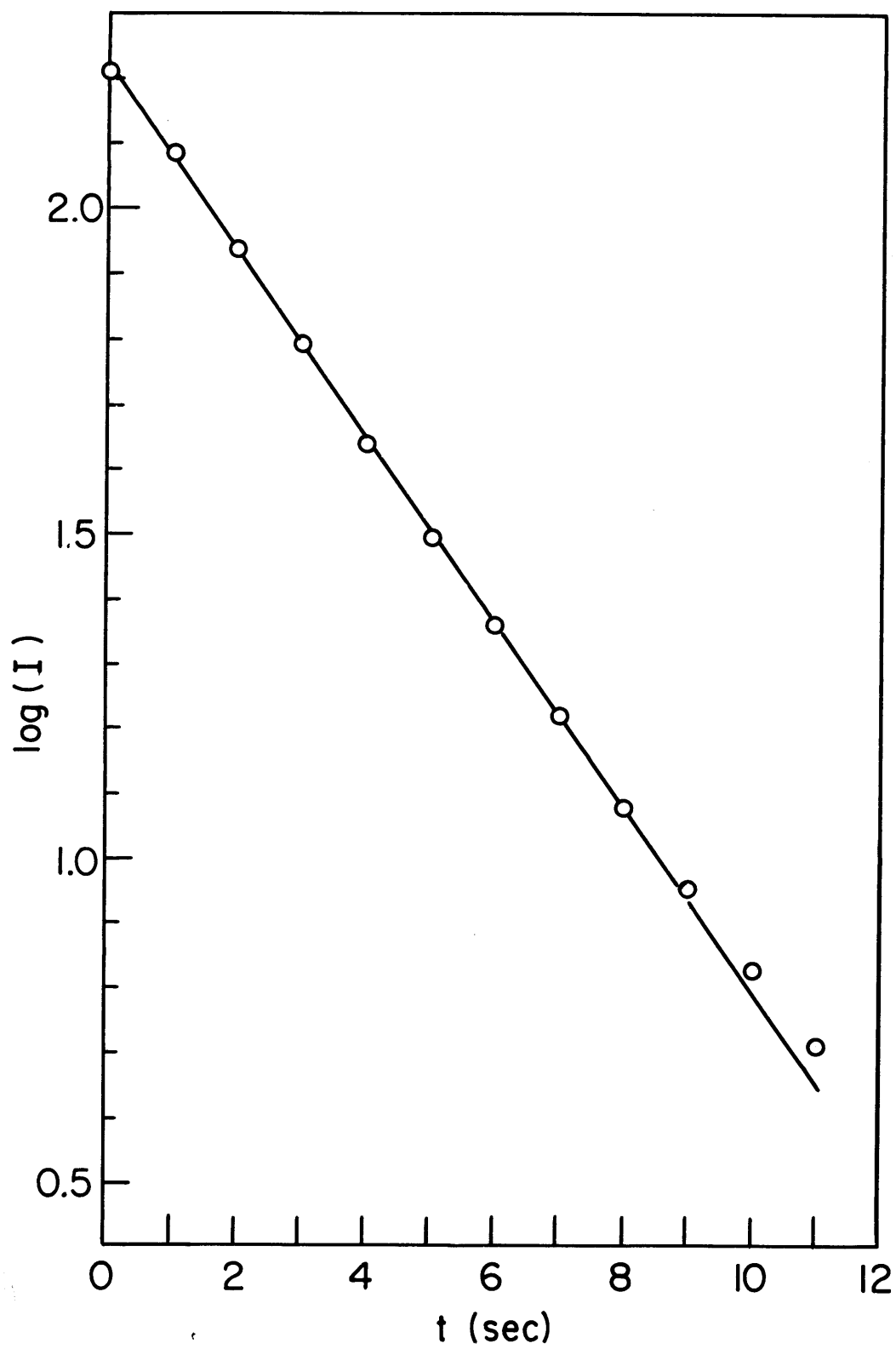


Figure III-23

Normalized CL Decay Curves for Various BTO
Concentrations

Key

Before Mixing:

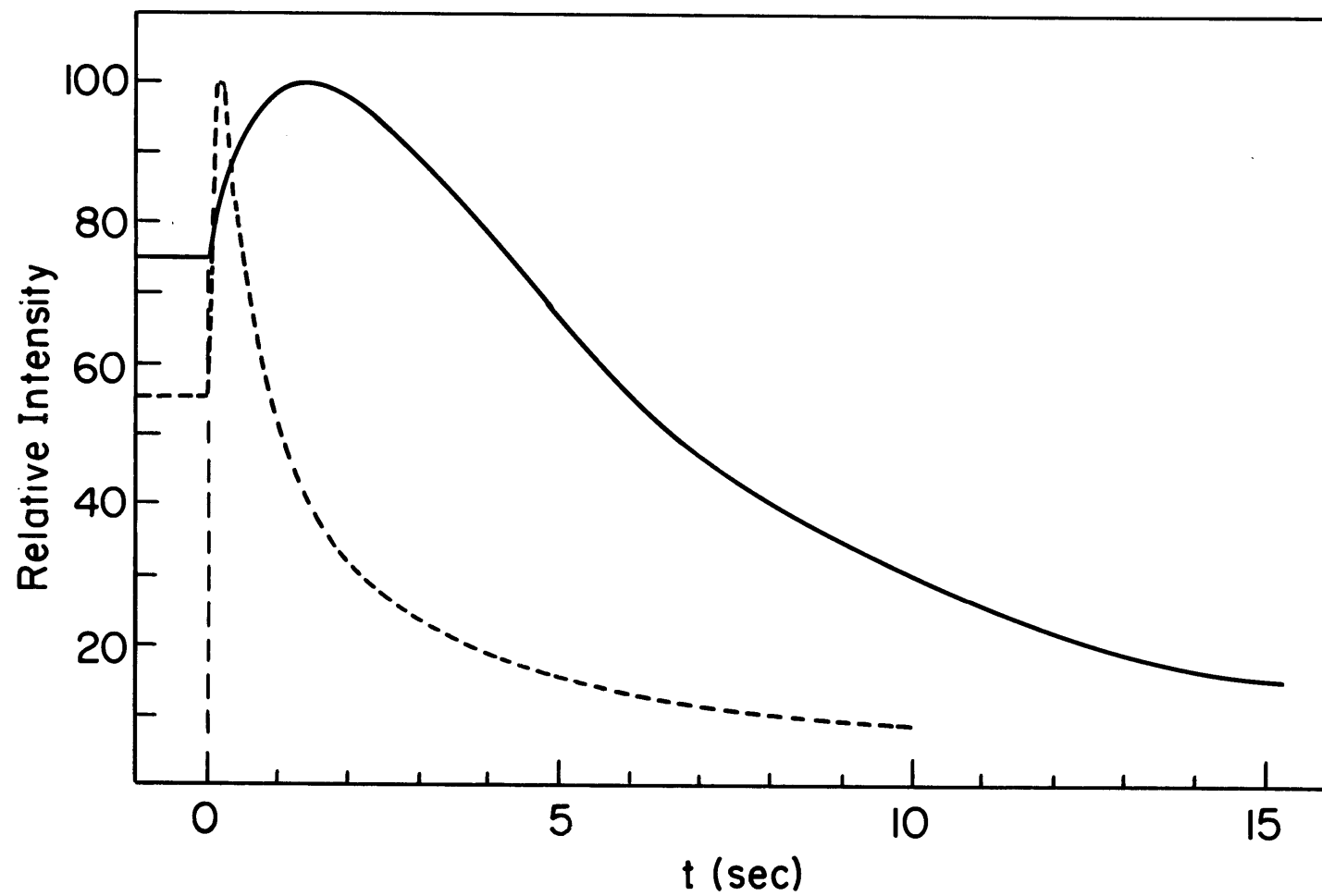
$$[\text{NaLUM}] = 1.0 \times 10^{-3}\text{M}$$

$$[\text{O}_2] = 2 \times 10^{-3}\text{M}$$

$$[\text{BTO}] =$$

$$\text{-----} \quad 6.8 \times 10^{-4}\text{M} \text{ (R}_{\text{FB}} = 10\text{K})$$

$$\text{—————} \quad 4.6 \times 10^{-5}\text{M} \text{ (R}_{\text{FB}} = 100\text{K})$$



($R_{FB} = 100K\Omega$). The very low signal for the lower concentration might not be accurate because of some decomposition of the BTO solution. As can be seen in Figure III-23, even at the very low BTO concentration the CL decay curve still shows a maximum at 1.5 seconds. A plot of $\log(I)$ versus t for this curve is shown in Figure III-24. The plot becomes linear after 3.0 seconds and has a $K' = 6.8 \times 10^{-2} \text{ sec}^{-1}$.

Experiments using various oxygen concentrations and constant NaLUM ($1.0 \times 10^{-3} \text{ M}$) and BTO ($1.9 \times 10^{-3} \text{ M}$) concentrations showed that the maximum of the CL decay curve did not disappear for low oxygen concentrations. However, for an oxygen concentration of about $2 \times 10^{-4} \text{ M}$ the decay curve (maximum at 0.6 sec) was pseudo-first order after 2 seconds with $K' = 1.5 \times 10^{-1} \text{ sec}^{-1}$. The $\log(I)$ versus t plot for this decay curve is shown in Figure III-25. For this oxygen concentration the reaction was over in about 10 seconds.

2. CL Decay in Mixed Solvents

Stopped-flow CL decay curves were also recorded for the solutions used to obtain the CL spectra in the mixed solvents 1-7 (see Section III-A-2). These decay curves all showed maxima, and the relative changes in the steady-state and maximum intensities of the decay curves in these mixed solvents paralleled the relative changes in the intensity of the CL spectrum shown in Table III-4. Increasing the concentration of water in the mixed solvents greatly increased the time required for the initial CL intensity to decay. For example, in DMSO using reactant concentrations similar to those used in the mixed solvent study, the

Figure III-24

Plot of Log (I) Versus Time for the CL Decay

Curve Shown in Figure III-23 ($[BTO] = 4.6 \times 10^{-5}M$)

$$k' = 6.8 \times 10^{-2} \text{sec}^{-1}$$

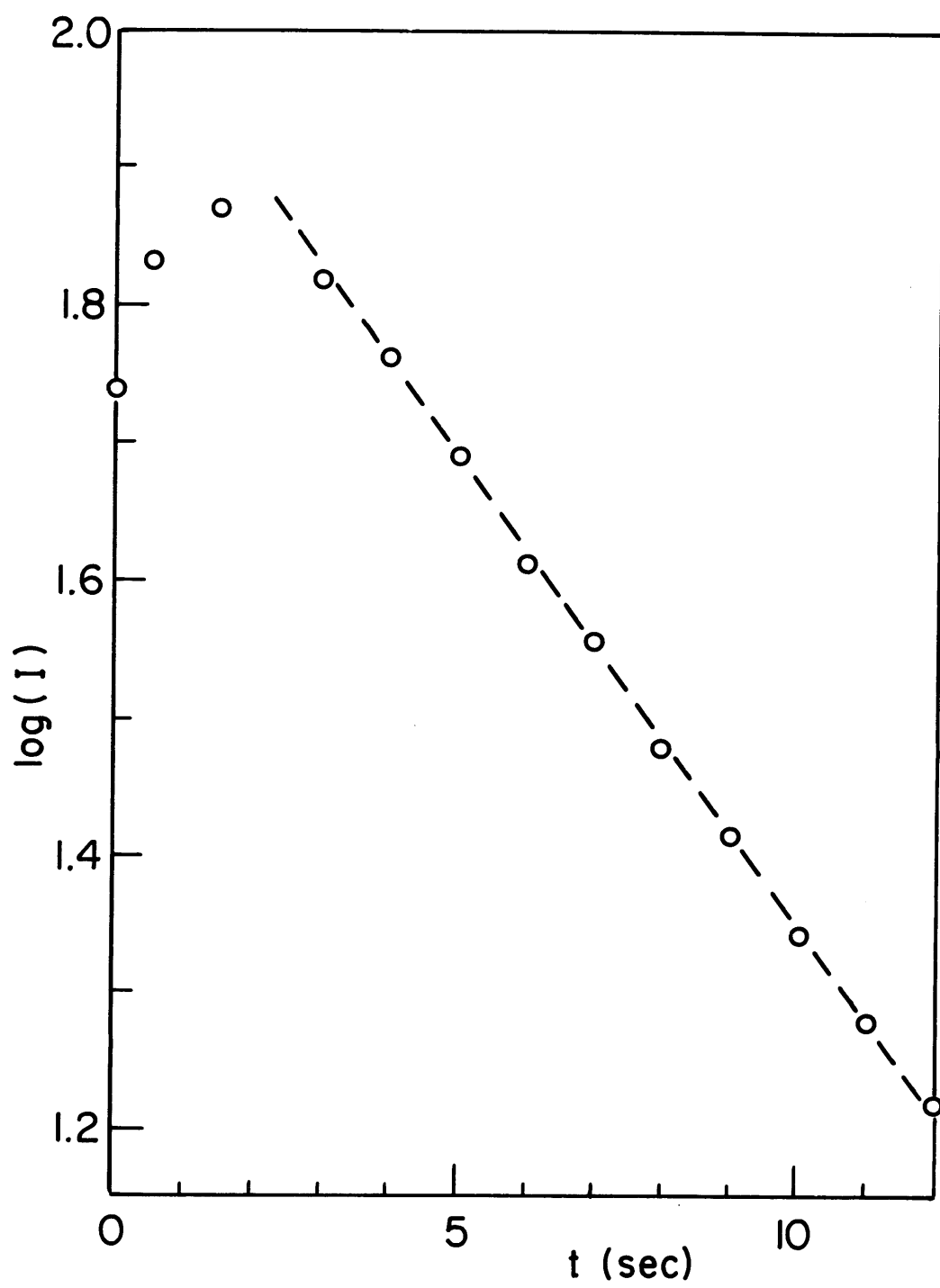


Figure III-25

Plot of Log (I) Versus Time for the CL Decay
Curve Obtained Using Low Oxygen Concentration

Key

Before Mixing:

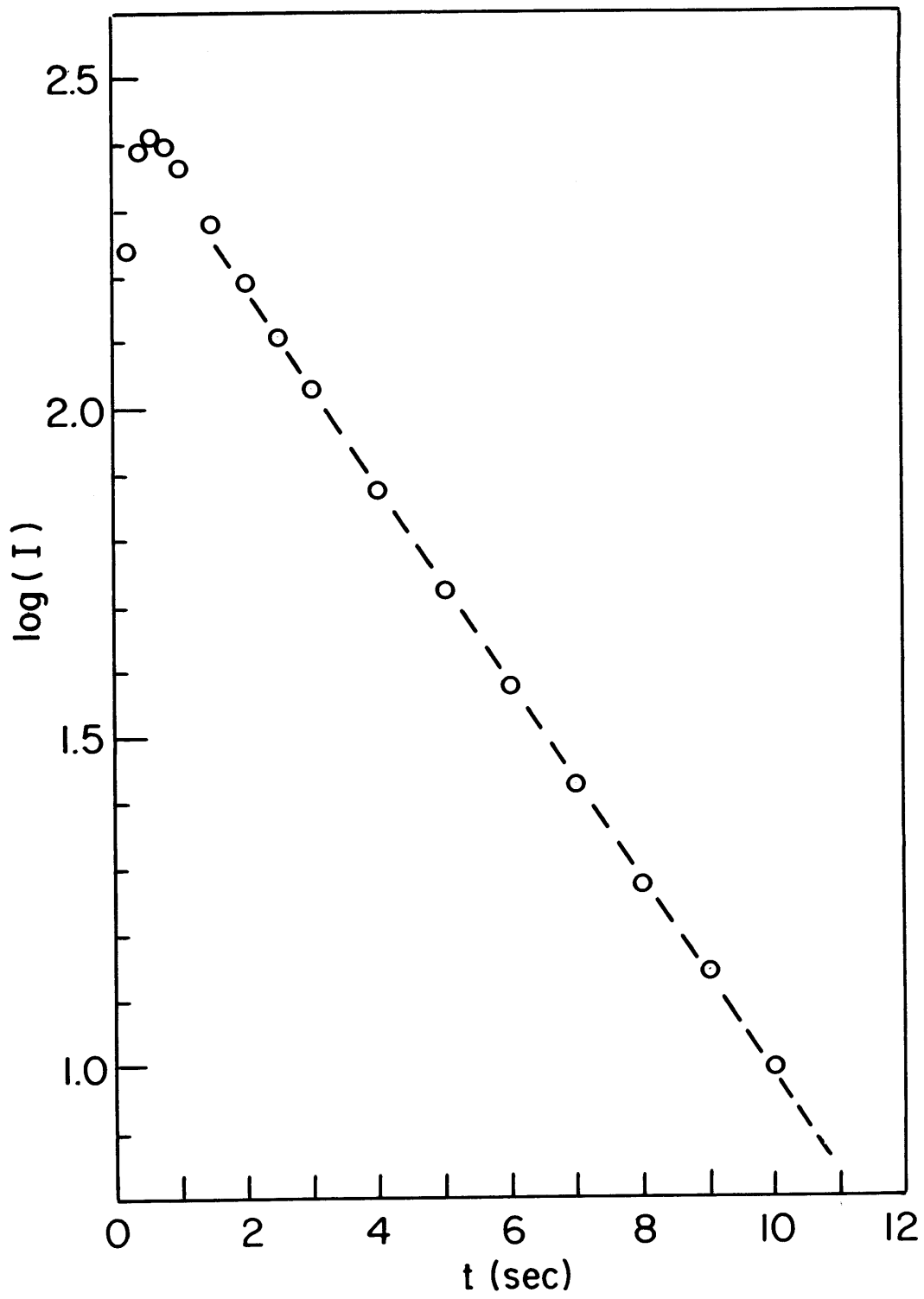
$$[\text{NaLUM}] = 1.0 \times 10^{-3}\text{M}$$

$$[\text{BTO}] = 1.9 \times 10^{-3}\text{M}$$

$$[\text{O}_2] = 2 \times 10^{-4}\text{M}$$

$$R_{\text{FB}} = 51\text{K}$$

$$k' = 1.5 \times 10^{-1}\text{sec}^{-1}$$



time required for the maximum CL intensity to decay to one-half, $t_{1/2\max}$, is approximately 1.0 second. In mixed solvents 1, 2, 3, and 4 the $t_{1/2\max}$ values are approximately 5 seconds, 10 seconds, 21 seconds, and 4.2 minutes, respectively. The CL intensity in mixed solvent 4 decayed to zero in about 20 minutes. The decay times for the CL in mixed solvents 5, 6, and 7 were so long that it was impractical to measure them.

The decay curves in mixed solvents 4-7 were particularly interesting. For these curves the steady-state intensity was nearly zero and when the flow was stopped, the intensity of the CL increased to the maximum value in 5 to 10 seconds and then the very slow CL decay began. The initial rise in the CL intensity in mixed solvent 5 is shown in Figure III-26.

Figure III-26

Initial Rise in the CL Decay Curve Obtained
Using Mixed Solvent 5

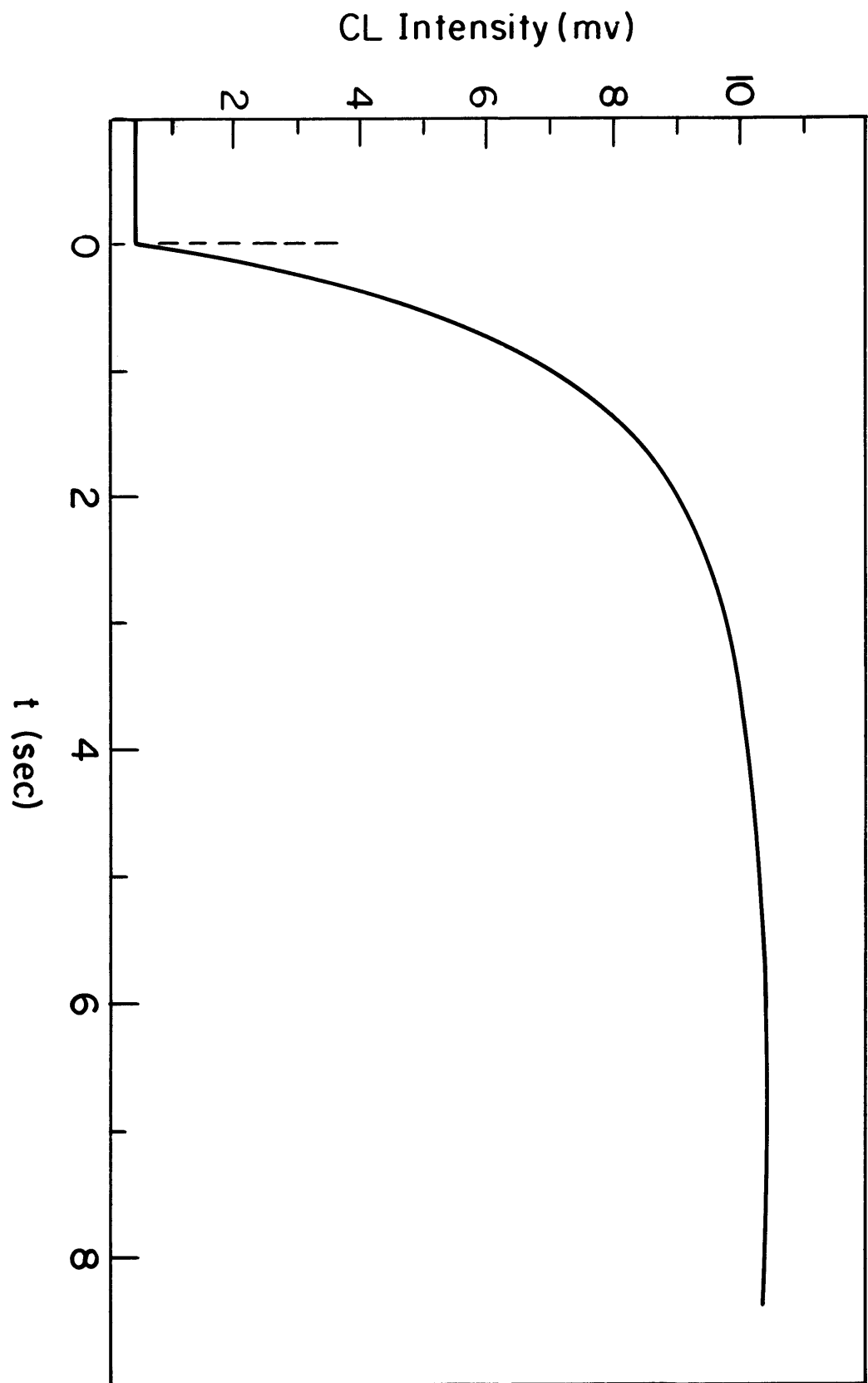
Key

Before Mixing:

$$[\text{NaLUM}] = 3.0 \times 10^{-4}\text{M}$$

$$[\text{KOH}] = 5 \times 10^{-2}\text{N}$$

$$[\text{O}_2] = 2 \times 10^{-3}\text{M}$$

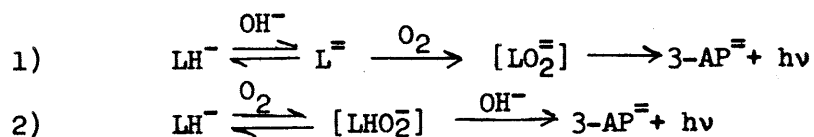


IV. Discussion

A. Luminol Study

1. Nature of the Reactive Species

The observations by White et al. (14) that CL is observed only from solutions of luminol which contain slightly more than one mole of base and that two moles of base are consumed in the overall luminol reaction suggest, but do not prove, that the first step in the luminol reaction in DMSO involves the dianion and oxygen. The reaction of oxygen with the monoanion, and the subsequent reaction of this species with the second mole of base must also be considered. These reaction schemes are shown below.



Since both of these reaction schemes require oxygen and the second mole of base to produce CL, distinguishing between them presents an interesting problem.

The results of White et al. (14) discussed above were obtained using NaOH as the base in the luminol reaction. Since NaOH is insoluble in dry DMSO, these workers were using DMSO which contained a considerable quantity of water. Preliminary experiments in this laboratory indicated that small amounts of water in DMSO greatly reduced the intensity of the luminol CL, i.e. the reaction rate. It was felt that a detailed spectroscopic study of luminol and the

luminol CL in basic DMSO and DMSO-water mixed solvents would provide additional information which would help identify the reacting species in the luminol reaction.

Absorption spectra of carefully vacuum-degassed solutions of NaLUM in basic DMSO (using BTO) have shown the formation of a new species which is in equilibrium with the monoanion. This species is believed to be the luminol dianion. This conclusion is supported by the fact that the PD dianion was also formed in deoxygenated solutions of NaPD containing excess BTO. Also, the absorption spectra of the PD dianion and the luminol dianion in DMSO are very similar (see Figures III-2 and III-16). Titration of the luminol monoanion ($1 \times 10^{-4}M$) with a concentrated solution of BTO showed that a base concentration of about $10^{-3}M$ was sufficient to completely shift the equilibrium to the dianion.

In aqueous solution under similar conditions the absorbing species is the monoanion (see Figure III-1). At this base concentration formation of the dianion in aqueous solution is not expected since the second ionization constant of luminol has been estimated to be about 10^{-13} (30). Considering the magnitude of this ionization constant, White et al. (14) erroneously concluded that even in DMSO only a low concentration of the dianion would be present in the reaction mixture.

Removal of the second proton in DMSO can occur because in an aprotic solvent, such as DMSO, bases are not solvated and can exhibit their intrinsic basicity. Therefore, bases such as hydroxides

and alkoxides are extremely strong in DMSO (31) (32). In aqueous solution, the leveling effect of the water limits the base strength to the basicity of the hydroxide ion, and this is not a strong enough base to remove the second proton.

Evidence for the formation of LHC_2^- could not be found. The absorption spectrum of a deoxygenated solution of the monoanion in DMSO was identical to the spectrum obtained when the solution was saturated with oxygen. These solutions showed no CL. If LHO_2^- and LH^- are in equilibrium, it is apparent that the equilibrium lies far to the left in reaction scheme 2. Therefore, it seems unlikely that such low concentrations of LHO_2^- could account for the high initial CL intensity and the rapid CL decay (< 10 sec) observed when a DMSO solution of the monoanion is reacted with base and oxygen.

The spectroscopic evidence for the quantitative formation of the dianion in DMSO, and the fact that CL is observed only when luminol solutions containing some dianion (determined by absorption spectrum) are exposed to oxygen, indicate that the dianion is the key intermediate in the luminol chemiluminescent reaction in DMSO. Since the dianion is not formed in basic aqueous solution, it is understandable that aqueous solutions of luminol show no CL with just oxygen and base present while solutions of luminol in DMSO are strongly chemiluminescent under the same conditions. The need for oxygen and potassium ferricyanide or hydrogen peroxide to initiate the chemiluminescent reaction in aqueous solutions suggests that the first step in the reaction is probably an oxidation involving the removal

of a hydrogen atom from the monoanion. This mechanism is consistent with the results of Cormier and Prichard (13). These workers detected the intermediate formation of luminol radicals during the aqueous CL and concluded that the luminol radicals are necessary intermediates for the luminol chemiluminescence.

Further evidence for the participation of the dianion in the CL reaction is provided by the luminol absorption and CL spectra obtained in the basic DMSO-water mixed solvents. If the dianion is responsible for the initiation of the CL reaction and this species is present only in the DMSO environment, then the changes observed in the steady-state CL intensity as a function of mixed solvent composition should correlate with the changes observed in the dianion absorption spectrum, i.e. the dianion concentration in the corresponding mixed solvents.

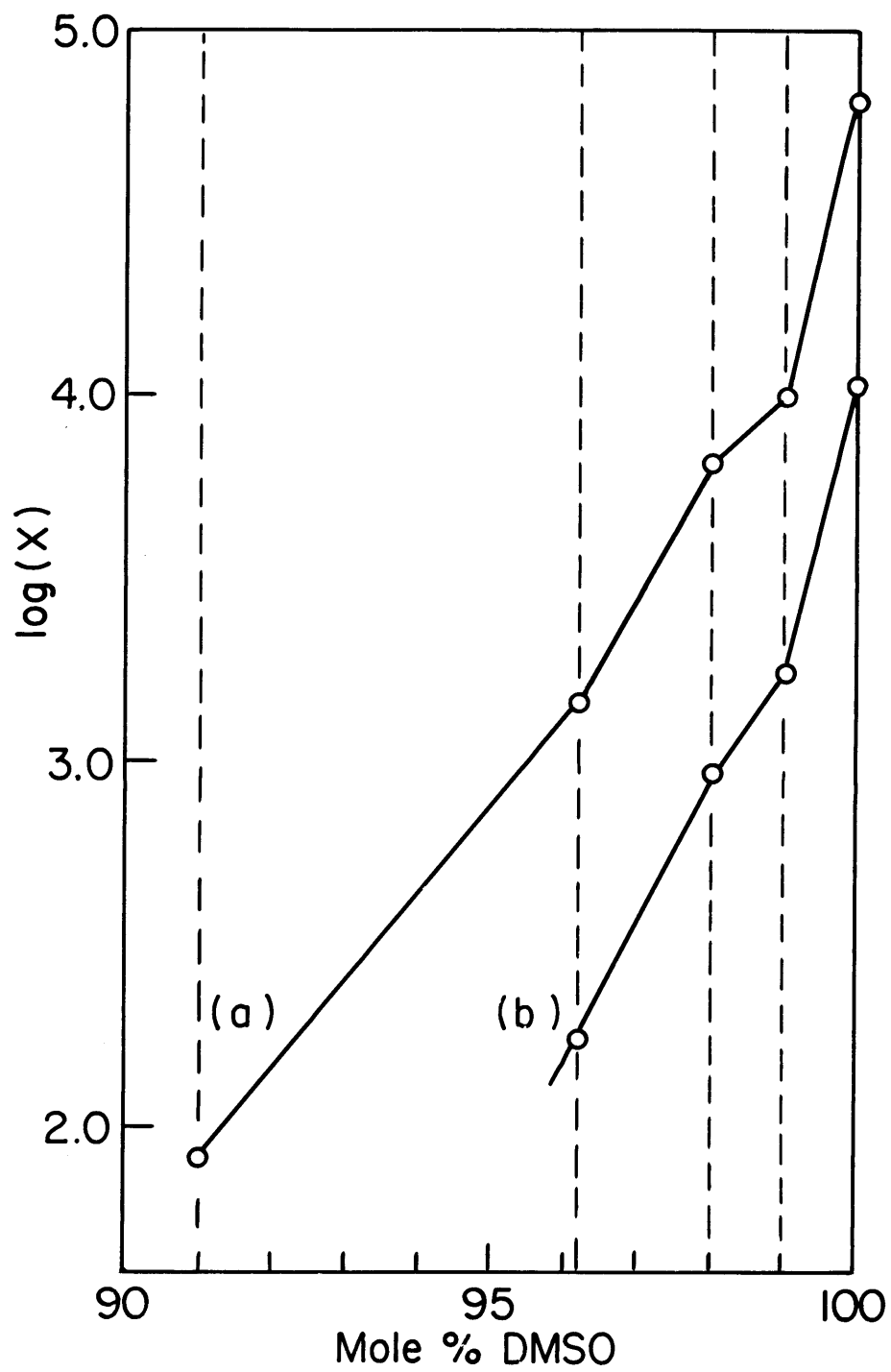
The effect of water on the stability of the dianion can be seen in Figure III-3 where the absorption spectra of NaLUM in basic DMSO and mixed solvents 1, 3, and 4 are shown. It is apparent from these spectra that very small amounts of water considerably reduce the concentration of the dianion by shifting the equilibrium in the direction of the monoanion. Solutions containing similar concentrations of NaLUM and base also showed marked decreases in CL intensities with increasing water concentration (see Table III- 4). Figure IV-1 shows a log plot of the relative CL intensity and relative concentration of the dianion versus the mole % of DMSO for DMSO and mixed solvents 1, 2, 3, and 4. The log plot was used only because of

Figure IV-1

Correlation of the Relative CL Intensity and
the Relative Concentration of the Luminol Dianion
as a Function of the Mixed Solvent Composition

Log (X) versus Mole % DMSO

Key-- Curve a: X = Relative CL Intensity
Curve b: X = Relative Concentration of
the Luminol Dianion



the large changes observed and no linear dependence is implied.

The concentration of the dianion present in a particular mixed solvent was calculated from the measured absorbance at 450 nm using the extinction coefficient measured for the dianion in DMSO at this wavelength. This particular wavelength was chosen because only the dianion absorbs at 450 nm. Although the solutions of NaLUM and base used to obtain the absorption spectra in mixed solvents 4 and 5 showed weak CL when exposed to oxygen, the monoanion-dianion equilibrium in these solvents had shifted almost completely to the monoanion, and the dianion absorbance at 450 nm became too small to measure.

As can be seen in Figure IV-1, the rapid decrease in CL intensity with increasing amounts of water parallels the decrease in the concentration of the dianion. This direct correlation between the decrease in CL intensity and dianion concentration clearly implicates the dianion as the critical intermediate in the luminol CL reaction (reaction scheme 1).

For the mixed solvents studied the strong dependence of the CL intensity on the base concentration is also consistent with the conclusion that the dianion is the reacting species. Increasing the base concentration resulted in increased absorption in the region where the dianion absorbs and a commensurate increase in the CL intensity. For example, although NaLUM ($1 \times 10^{-4}M$) in mixed solvent 6 shows no emission with $2.5 \times 10^{-3}N$ KOH and oxygen present, a weak, long-lived CL is observed in this solvent when the base concentration is increased by an order of magnitude. The increased base concen-

tration shifts the monoanion-dianion equilibrium enough to form a very small amount of the dianion and the chemiluminescent reaction can take place.

It has recently been reported by Matheson and Lee (33) that BTO reacts with oxygen in DMSO to form superoxide, $\text{O}_2^{\cdot -}$. This raises the question of whether the formation of the luminol dianion is really necessary for CL. When a NaLUM solution containing oxygen is treated with excess BTO, the BTO could be reacting with the oxygen to form $\text{O}_2^{\cdot -}$ which then reacts with the luminol monoanion to produce CL. However, the results of the present investigation have shown that the luminol dianion is produced quantitatively in a deoxygenated solution of NaLUM when an excess of BTO is present. Therefore, if $\text{O}_2^{\cdot -}$ is formed when a solution of the NaLUM is reacted with BTO and oxygen, it is probably reacting with the luminol dianion rather than the monoanion.

An experiment was run to determine if $\text{O}_2^{\cdot -}$ is a major reactant in the chemiluminescent reaction in DMSO. In this experiment solutions of NaLUM in DMSO and BTO in DMSO were placed in separate arms of a special cell which could be vacuum-degassed. Oxygen was bubbled through the BTO solution for a few seconds, the cell was then quickly connected to a vacuum system and both solutions were vacuum-degassed three times. If the $\text{O}_2^{\cdot -}$ is formed in the BTO solution, it should remain in solution while the oxygen gas is removed by the vacuum degassing. When these degassed solutions were poured together under vacuum, no CL was observed. This suggests

that either the $O_2^{\cdot -}$ was simply not present, or it was present but did not react with the luminol dianion.

2. Absorption Spectra of PD and Luminol

A comparison of the absorption spectra of PD and luminol in ethanol (Figure III-14) and in DMSO (Figure III-13) shows that substitution of the amino group in the 5-position of PD causes a new band to appear in the PD absorption spectrum. This results from the delocalization of the electrons of the amino group over the aromatic ring. Since the long wavelength band in the PD spectrum most likely results from a charge-transfer (CT) transition in which the movement of charge is from the ring to the carbonyl group, the n-electrons of the amino group would be expected to interact strongly with the π -electrons of the ring. From the nature of the luminol absorption spectrum, it appears that this interaction produces a splitting of the CT state of PD.

Depending on the energy of the n-electrons of the amino group relative to the π -electrons of the PD, two types of interactions would be expected (34). These are illustrated in Figure IV-2. In Figure IV-2a the n-electrons are above the π -electrons and the interaction of these two levels results in a lowering of the π -electrons and a raising of the n-electrons. The π^* level is affected very little by the n-electrons. The net effect would be to introduce a new CT band at a longer wavelength relative to the PD CT band.

If the n-electrons are below the π -electrons, as

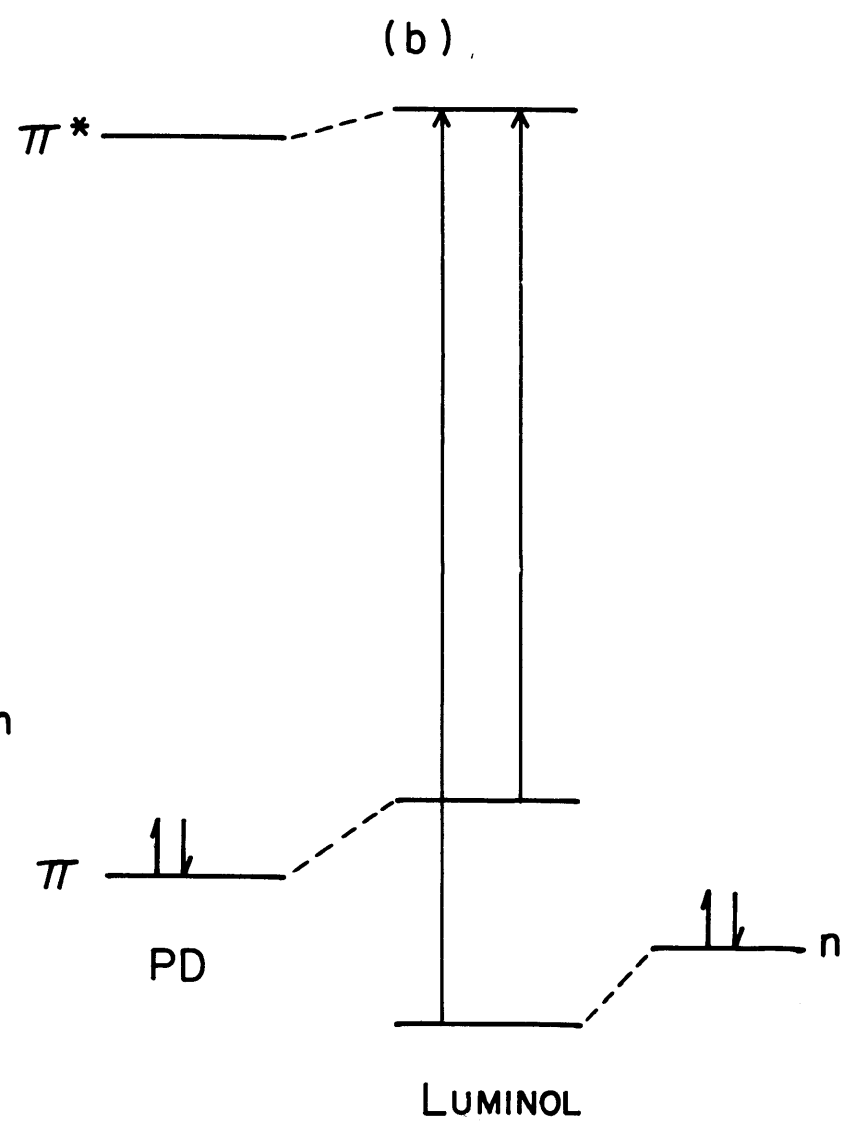
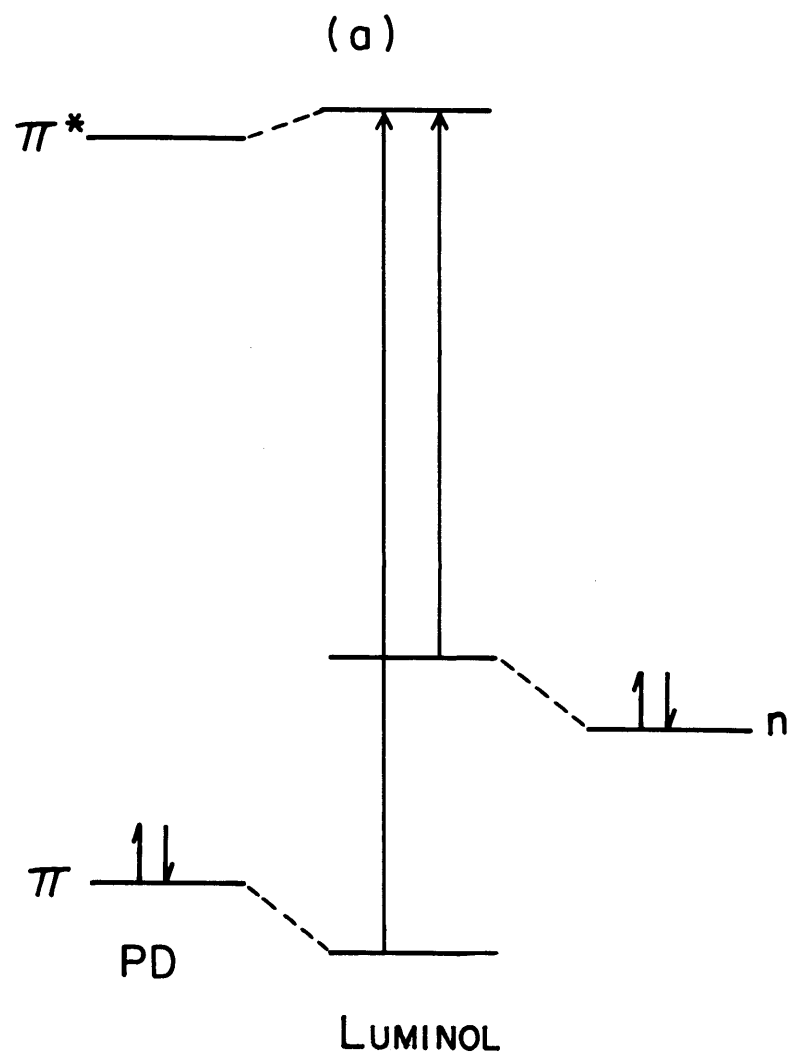
Figure IV-2

Diagram Showing the Interaction of the n-electrons
of the Amino Group with the π -electrons of PD

Key

Curve a: n-electrons above π -electrons

Curve b: n-electrons below π -electrons



illustrated in Figure IV-2b, the nature of the interaction would be to raise the π -electrons and lower the n-electrons. Again the π^* level is raised very little. This interaction of n-electrons and π -electrons would shift the PD CT band to longer wavelengths, and the short wavelength band would now be the new CT band.

Some evidence for the nature of the interaction responsible for the luminol spectrum is provided by the absorption spectra of PD, luminol, and their 2,3-dimethyl derivatives. These spectra in ethanol and DMSO are compared in Figures III-14 and III-13, respectively. The absorption data for these compounds in aqueous solution are tabulated in Tables III-5 and III-7. As can be seen in Table III-7, the PD and 2,3-DiMePD absorption maxima are shifted to longer wavelengths by 13 nm and 12 nm, respectively, when the solvent is changed from water to DMSO. The data in Table III-5 indicate that only the long wavelength bands of the luminol and 2,3-DiMeLUM absorption spectra show a solvent shift of this magnitude. The short wavelength bands are shifted only 2 to 3 nm. Thus, the nature of this solvent effect on the absorption spectra of PD, luminol, and their dimethyl derivatives suggests that the PD CT band is responsible for the long wavelength band in the luminol absorption spectrum. These results are supported by the shifts observed in the absorption spectra of PD and luminol upon dimethyl substitution. Dimethyl substitution of PD shifts the PD absorption maximum in each of these solvents to longer wavelengths (305 \rightarrow 311 nm in DMSO). A comparison of the luminol and 2,3-DiMeLUM absorption spectra in these solvents show that the

short wavelength band of luminol is shifted to shorter wavelength (297 \rightarrow 294 nm in DMSO) while the long wavelength band is shifted to longer wavelengths (360 \rightarrow 370 nm (band center) in DMSO) by the dimethyl substitution.

Since the 2,3-DiMePD absorption spectrum is shifted to longer wavelengths relative to the PD spectrum, the same shift would be expected for the PD CT band in the spectra of luminol and 2,3-DiMeLUM. Therefore, on the basis of the red-shift of the long wavelength band of the luminol spectrum upon dimethyl substitution and the solvent effects discussed above, it is concluded that the interaction of the amino group with the aromatic ring shifts the PD band to longer wavelengths (\sim 360 nm) and introduces a new CT band at about 300 nm. This is the type of interaction shown in Figure IV-2b.

In order for the interaction described above to occur, the geometry of the amino group with respect to the aromatic ring must be such that the n-electrons can interact with the π -system. The greatest interaction would be expected when the amino group is coplanar with the ring system. In the case of luminol, the positions of the amino and carbonyl groups provide an ideal situation for the formation of a six-membered ring and an intramolecular hydrogen-bond (H-bond) to stabilize the amino group in the coplanar configuration. The occurrence of a new band in the luminol absorption spectrum indicates that there is a strong interaction of the n- and π -electrons, and suggests the coplanarity of the amino group and aromatic ring. Some convincing evidence that this is indeed the case is the fact that

the absorption spectrum of 5-dimethylaminoPD (35) is essentially the same as the absorption spectrum of PD. The dimethylamino group in the 5-position is sterically hindered and cannot lie in the same plane as the aromatic ring. Therefore, the interaction of the n-electrons with the aromatic ring is minimized.

The absorption spectrum of 6-diethylaminoPD is also quite interesting. The complete absorption spectrum in ethanol ($\lambda_{\text{max}} = 284 \text{ nm}$ ($\epsilon = 23,708$)) is shown in Figure III-14. The absorption spectrum is similar in DMSO ($\lambda_{\text{max}} = 289 \text{ nm}$ ($\epsilon = 24,550$)). Although the diethylamino group in the 6-position is not sterically hindered from lying coplanar with the ring, it cannot be held in the coplanar configuration through intramolecular H-bonding as is the case for luminol. Therefore, it is free to rotate and the interaction of the n-electrons with the aromatic ring is weakened. From the shape (shoulders at about 310 nm in ethanol, 310 nm and 360 nm in DMSO) and intensity of the absorption spectrum, it appears that there are several transitions responsible for the spectrum. Evidently, the weakened interaction still produces two CT states, but they are very close together and result in one intense absorption band. As expected, the absorption spectrum of 6-dimethylaminoPD ($\lambda_{\text{max}} = 284 \text{ nm}$ ($\epsilon = 25,000$) in tetrahydrofuran) (35) is very similar (both in intensity and shape) to the spectrum of 6-diethylaminoPD.

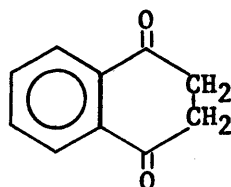
3. A Study of Keto-Enol Tautomerism

A detailed study of the spectral characteristics of

luminol in DMSO and water is complicated, not only by the various acid-base equilibria involved, but also by the ability of this molecule to exhibit keto-enol tautomerization. Several investigators (22) (23) have studied the keto-enol tautomerization in the phthalhydrazides, but their results are difficult to interpret. In the present investigation an effort was made to examine the tautomeric behavior of luminol in DMSO and water by studying the absorption characteristics of PD and several methyl and dimethyl derivatives.

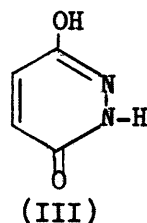
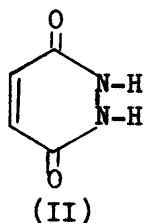
a. PD

The absorption characteristics of PD, MePD, and 2,3-DiMePD in water and DMSO are shown in Table III-7. The similarity of the absorption spectra of PD, MePD, and 2,3-DiMePD in water ($\lambda_{\text{max}} = 292, 298, 299 \text{ nm}$, respectively) (complete spectra of PD and 2,3-DiMePD in ethanol shown in Figure III-14) indicate that PD exists as the diketo form in this solvent. Since the shift of the 2,3-DiMePD absorption maxima in changing the solvent from water to DMSO ($\Delta\lambda = 12 \text{ nm}$) is similar to that observed for PD ($\Delta\lambda = 13 \text{ nm}$), one would have to conclude that the same species is present in DMSO. The solvent shifts observed for these compounds in water reflect the expected changes in H-bonding. The similarity of the absorption characteristics of (I)



(I)

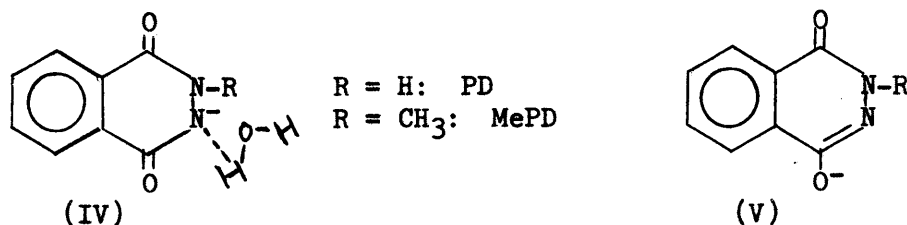
($\lambda_{\text{max}} = 295 \text{ nm}$ ($\log \epsilon = 3.2$), 255 nm ($\log \epsilon = 4.0$), and 225 nm ($\log \epsilon = 4.5$)) (36) and PD also support the diketo structure for PD. Infrared data to verify the diketo structure could not be obtained because of the low solubility of these compounds in the normal infrared solvents. The crystalline form of PD is apparently in the diketo structure since the infrared spectrum in KBr showed what appeared to be the carbonyl band at 1640 cm^{-1} and no OH band. The carbonyl band occurs in the region expected for this type of compound (37). It is interesting to note that maleic hydrazide (II) which should show similar



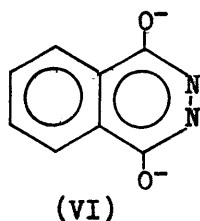
acid-base and tautomeric behavior has been shown by Miller (38) (39) to exist in the mono-enol structure (III). This conclusion has been supported by NMR studies (40).

As can be seen in Table III-8, the monoanions of both PD and MePD show very large shifts in absorption maxima when the solvent is changed from water to DMSO ($310 \text{ nm} \rightarrow 364 \text{ nm}$ and $313 \text{ nm} \rightarrow 367 \text{ nm}$, respectively). Since the molecular form of PD exists in the diketo structure in both water and DMSO, this shift cannot be attributed to a tautomeric change. The fact that the absorption spectra of PD in DMSO-water mixed solvents show no isosbestic points as the absorption maximum gradually shifts to shorter wavelengths with increasing amounts of water clearly indicates that the observed spectral

shifts do not result from the formation of a new species. Although the dramatic shift in the absorption maximum is much larger than expected for changes in hydrogen-bonding, it apparently is a solvation effect. A possible explanation is that after the dissociation of a proton, the charge remains localized on the nitrogen in water because of the strong H-bonding (IV) while in DMSO the charge becomes localized on the oxygen (V).



Since the absorption spectrum of the PD dianion in DMSO is shifted to even longer wavelengths ($\lambda_{\text{max}} = 402 \text{ nm}$), this species probably becomes completely aromatic (VI).

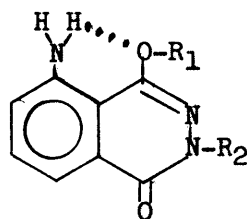


b. Luminol

The similarities of the absorption characteristics of luminol, 2-MeLUM, 3-MeLUM, and 2,3-DiMeLUM in aqueous solution (see Table III-5) suggest that luminol exists in the diketo form in water. The changes observed for the absorption spectrum of luminol in DMSO-water mixed solvents (Figure III-7) appear to result from changes in solvation rather than a shift in tautomeric equilibrium. Similar

spectral shifts are observed for PD in DMSO and water. This indicates that luminol also has the diketo structure in DMSO. The existence of the diketo structure in DMSO is supported by the fact that the absorption spectra of 2-MeLUM, 3-MeLUM, and 2,3-DiMeLUM in DMSO (see Table III-5 and Figures III-10, III-11, and III-13) are not dramatically different from the luminol spectrum.

However, there is also some evidence which indicates that the situation is not so simple. For instance, it is interesting to note the very similar nature of the absorption of spectra of luminol, 2-MeLUM, and 2-Me-4-MeOLUM in DMSO shown in Figure III-15. Although not shown, the spectrum of 4-MeOLUM also is very similar in shape to these spectra. These results suggest that perhaps the luminol exists in the mono-enol form in DMSO and that the enolization occurs at the carbonyl next to the amino group (I).



$R_1 = R_2 = H$: Luminol
 $R_1 = H, R_2 = CH_3$: 2-MeLUM
 $R_1 = R_2 = CH_3$: 2-Me-4-MeOLUM
 $R_1 = CH_3, R_2 = H$: 4-MeOLUM

(I)

If this is the case, then either the small spectral shifts shown by luminol in the mixed solvents were indeed the result of a change in tautomeric equilibrium, or the mono-enol form also exists in water. The latter is a possibility since the spectrum of 2-Me-4-MeOLUM in water closely resembles the aqueous luminol spectrum.

The only absorption spectra which might be considered

significantly different from the luminol spectrum in both water and DMSO were the spectra of 1-MeOLUM (Figure III-12 in DMSO) and 1-MeO-3-MeLUM (see Table III-5). On the basis of these spectral differences, one can at least conclude that enolization is not occurring at the carbonyl in the 1-position of luminol.

The absorption spectra of the monoanions of luminol and some of its methyl derivatives in water and DMSO (Table III-6 and Figures III-2, III-10, III-11, and III-12) are complicated by solvation effects similar to those observed for the PD monoanion. As a result, these spectra provide little information concerning the tautomeric behavior of luminol and will not be discussed. Since the spectra of these monoanions have not been reported, they are included for future reference.

The question of whether the same tautomeric form of luminol exists in DMSO and water was not the main concern of this investigation. Nevertheless, it was felt that a study of the absorption spectra of luminol and some of its methyl derivatives might provide some useful information concerning the tautomeric behavior of luminol. The results of this investigation clearly indicate that ultraviolet absorption spectroscopy is not the technique to use to study this particular system. If there are different tautomeric species in these solvents, it is apparent that their absorption characteristics are very similar, and any spectral changes which might result from a shift in the tautomeric equilibrium are obscured by solvation effects.

As was the case for PD, solubility problems prevented

a detailed study of luminol by infrared spectroscopy. Since the infrared spectra of both luminol and NaLUM in KBr showed what appeared to be carbonyl bands at 1650 cm^{-1} and 1640 cm^{-1} , respectively, and no OH bands, the crystalline forms of these compounds must exist in the diketo structure. An explanation of the tautomeric behavior of luminol in solution will obviously have to be found by some other technique. NMR and Raman spectroscopy might provide some answers although the solubility of these compounds in useful solvents will continue to be a problem.

4. Absorption Spectra of NaLUM in Basic DMSO-Water Mixed Solvents

The complex nature of the solvation effects occurring in DMSO and water can be appreciated by comparing the absorption spectra of NaLUM in basic DMSO-water mixed solvents shown in Figures III-3, III-4, III-5, and III-6. Depending on the mole % water in a particular solvent, one would expect to see a composite spectrum of the monoanion and dianion spectra along with the associated solvent shifts. Since the dianion is not observed in water, the contribution of the dianion to the absorption spectrum should be significant only for very low water concentrations.

As can be seen in Figure III-3, small amounts of water decrease the concentration of the dianion and shift the absorption spectrum to shorter wavelengths with increased absorption around 330 nm for mixed solvent 3. A comparison of the absorption spectra of the

luminol monoanion and dianion in DMSO (Figure III-2) and the monoanion in water (Figure III-1) shows that there is no way these spectra can be combined to show the type of absorption spectrum observed in mixed solvent 3. Since the absorption spectrum of just the monoanion in mixed solvent 3 is very similar to the monoanion spectrum in DMSO, the origin of the increased absorption at 330 nm is particularly puzzling. There is no apparent distortion of this absorption spectrum by solvent decomposition. Also, several different solutions of NaLUM in mixed solvent 3 gave the same spectrum.

The absorption spectra of NaLUM in mixed solvents 4 and 5 (Figure III-4) are similar to the monoanion spectrum in DMSO. These solutions chemiluminesce weakly when exposed to oxygen indicating there is also a very low concentration of the dianion present. Since no CL is observed from NaLUM in basic mixed solvent 6, only the monoanion is present.

The spectra shown in Figure III-5 for NaLUM in mixed solvents 6, 8, and 9 show that the development of the bands at 310 nm and 360 nm might result from a shift in an equilibrium (isosbestic points at about 280, 320, 340, and 380 nm). This suggests that perhaps different tautomeric forms of luminol exist in the DMSO and water environments. The changes in the spectra shown in Figure III-6 for mixed solvents 10, 11, and 12 are characteristic of a solvent shift and result from the increased H-bonding expected at these higher water concentrations.

B. 3-Aminophthalic Acid Study

1. Nature of the Emitting Species

Since the purpose of this investigation was to examine the spectral characteristics of 3-APA under conditions necessary for luminol CL, this part of the discussion will consider only the excited-states of $3\text{-AP}^=$ and the spectral changes observed in water and DMSO.

In aqueous solution the ground-state of the $3\text{-AP}^=$ is most likely solvated through the formation of intermolecular hydrogen-bonds (H-bonds) with the solvent. This interpretation is consistent with the blue shift in the dianion absorption spectrum when the solvent is changed from DMSO ($\lambda_{\text{max}} = 312 \text{ nm}$; no H-bonding) to water ($\lambda_{\text{max}} = 303 \text{ nm}$). This shift (1000 cm^{-1} , $\sim 2.8 \text{ kcal}$) is the order of magnitude expected for H-bonding effects (41) (42). Similar shifts are also observed for 4-aminophthalate and o-, m-, and p-aminobenzoates (see Table III-11).

The large Stokes' shifts observed for the fluorescence of $3\text{-AP}^=$ in both water ($303 \text{ nm} \rightarrow 425 \text{ nm}$) and DMSO ($312 \text{ nm} \rightarrow 490 \text{ nm}$) indicate that the equilibrium excited-state of $3\text{-AP}^=$ differs significantly from the "Franck-Condon" excited-state in these solvents. Since the charge distribution in the excited-state of $3\text{-AP}^=$ is different from that of the ground-state, solvent reorientation will occur following excitation. It is apparent from the magnitude of the Stokes' shift for $3\text{-AP}^=$ that either the equilibrium arrangement of solvent molecules about the excited $3\text{-AP}^=$ is quite different from that of the ground-

state in these solvents, or there is a change in the $3\text{-AP}^{\text{=}}$ itself following excitation, i.e. an excited-state equilibrium.

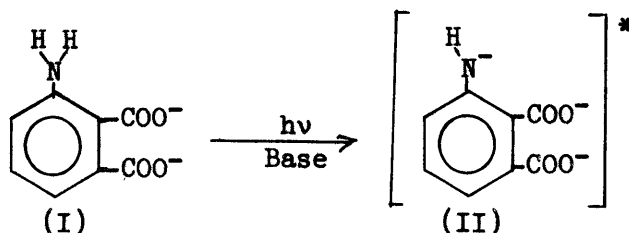
In the mixed solvents where two bands are observed for the $3\text{-AP}^{\text{=}}$ fluorescence (see Figure III-18), it is interesting to note that the short wavelength emission (~ 410 nm) is blue-shifted relative to the fluorescence spectrum in water. White and Bursey (15) have reported the two emission bands in DMSO-water mixtures, but indicated that the peak positions are unchanged from the values in the pure solvents. They attributed the occurrence of two emission peaks to the formation of two different kinds of excited-state molecules: excited $3\text{-AP}^{\text{=}}$ H-bonded to water and excited $3\text{-AP}^{\text{=}}$ not bonded in this way (DMSO). The results of the present study suggest that this explanation is incorrect.

First of all, if the short wavelength band in the mixed solvents is the fluorescence spectrum of $3\text{-AP}^{\text{=}}$ in water (425 nm), then the effect of introducing a new band at longer wavelengths (490 nm) would be to shift the fluorescence maximum of the water spectrum in the direction of the long wavelength band. Since the band occurs at about 410 nm, it cannot be identified as the $3\text{-AP}^{\text{=}}$ fluorescence spectrum in water. Also, the absorption spectrum of $3\text{-AP}^{\text{=}}$ does not change for mixed solvents 1-8 while the short wavelength band is developing in the fluorescence spectrum, and the excitation spectra for both emission bands are the same as the absorption spectrum of $3\text{-AP}^{\text{=}}$ in DMSO. These results indicate that there is no change in the ground-state of $3\text{-AP}^{\text{=}}$ in these mixed solvents and strongly suggest that

two different excited-states of $3\text{-AP}^=$ exist in DMSO. The water in the mixed solvents has changed the $3\text{-AP}^=$ excited-state which normally exists in the DMSO environment, and it is this new excited-state which is responsible for the short wavelength emission in the mixed solvents.

The fluorescence data tabulated in Table III-11 for 4-aminophthalate and m-aminobenzoate in water and DMSO show that the DMSO band is shifted to shorter wavelengths relative to the water band. It can also be seen in Table III-11 that o-aminobenzoate shows the same behavior as $3\text{-AP}^=$ with fluorescence maxima at 400 nm in water, 475 nm in DMSO, and 380 nm and 475 nm in mixed solvents. In view of the behavior of 4-aminophthalate and m-aminobenzoate one would expect the fluorescence spectrum of $3\text{-AP}^=$ in DMSO to occur at shorter wavelengths than the fluorescence spectrum in water. Since the short wavelength band of $3\text{-AP}^=$ in the mixed solvents is blue-shifted relative to the water spectrum, it is possible that the 410 nm band is the expected emission from the $3\text{-AP}^=$ excited-state in the DMSO environment and that a change in the $3\text{-AP}^=$ following excitation produces a "new" excited-state which is responsible for the long wavelength band (490 nm). The observation of the same type of long wavelength emission for o-aminobenzoate suggests that the adjacent amino and carboxylate groups are necessary for the formation of the "new" excited-state in DMSO.

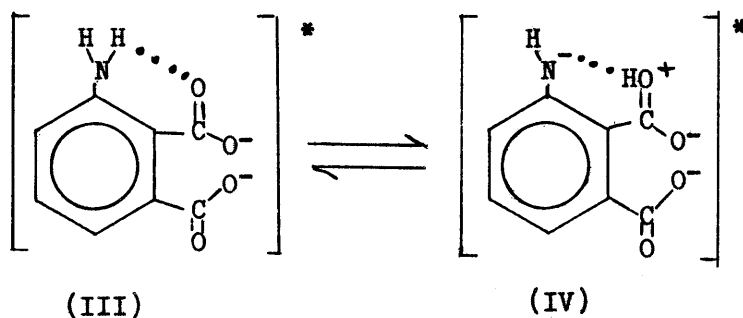
It is well known that pK's can change significantly in the excited-state (43). A possible explanation for the abnormally large Stokes' shift in DMSO could be the formation of the trianion (II) after excitation.



Some evidence which might support this is the observation that the fluorescence quantum yield, ϕ_f , for 3-AP⁼ in aqueous solution decreases above pH \approx 12 with no apparent change in the absorption spectrum (11). Förestér (44) reported the formation of a new fluorescence band for 2-naphthylamine at pH \approx 14 which he attributed to the loss of a proton from the amino group. However, since it was found in the present investigation that the intensities of the two fluorescence bands observed for 3-AP⁼ in mixed solvent 7 were independent of pH up to pH \approx 11, and since 4-aminophthalate and m-aminobenzoate do not show long wavelength emission bands similar to those observed for 3-AP⁼ and o-aminobenzoate, it is unlikely that the trianion is responsible for the 3-AP⁼ emission band at 490 nm in DMSO.

Recent studies have shown evidence that intermolecular H-bonding can be weakened and intramolecular H-bonding strengthened in the excited-state (45) (46). In DMSO intermolecular H-bonding would not be significant for either the ground- or excited-states of 3-AP⁼. However, the adjacent amino and carboxylate groups provide for six-membered ring formation via an intramolecular H-bond. The fact that the amino group becomes more acidic and the carboxylate group

becomes more basic in the excited-state makes the formation of the intramolecular H-bond even more favorable in the excited-state in DMSO. Since DMSO has been reported to affect greatly pK's of acid-base groups in the ground-state (31) (47), the properties of amino and carboxylate groups of 3-AP[±] in the excited-state could possibly be affected to the extent that a proton can be transferred from the amino group to the carboxylate group. Therefore, it is postulated that the proton-transferred excited-state of 3-AP[±] (IV) is responsible for the emission at 490 nm in DMSO.



In the mixed solvents which show the development of the short wavelength band, the 3-AP[±] proton-transferred excited-state is in equilibrium with the 3-AP[±] excited-state without proton-transfer (III). The excited-state without proton transfer is still essentially in a DMSO environment and is responsible for the emission band at about 410 nm. Increasing the concentration of water in the mixed solvents makes the formation of the proton-transferred excited-state less favorable and, as a result, the short wavelength emission band develops at the expense of the 490 nm emission band.

This explanation is consistent with the fluorescence characteristics observed for o-aminobenzoate, m-aminobenzoate, and 4-aminophthalate in water and DMSO. As expected, only the o-aminobenzoate shows the long wavelength emission in DMSO. Additional evidence for the proton-transferred excited-state should have been provided by the fluorescence spectra of the isomeric dimethylaminobenzoates. However, since the compound of particular interest, o-dimethylaminobenzoate, showed no fluorescence in DMSO, these results were inconclusive.

Excited-state proton transfer has been used by Weller (48) to rationalize the unusually large Stokes' displacements in salicylic acid and its esters. Similar proton transfers have been reported for several naphthoic acids (49).

Proton transfer would not take place for 3-AP[±] (or o-aminobenzoate) in aqueous solution because a significant amount of intermolecular H-bonding would be expected to persist in the excited-state. After excitation the electron density on the amino group would be directed to the aromatic ring and in the direction of the carboxylate groups. Although this would decrease the H-bonding at the amino group, the increased electron density on the carboxylate groups would result in the strengthening of the H-bonding at this part of the molecule. Although proton transfer does not take place, this stabilization of the excited-state via increased H-bonding accounts for the large Stokes' shift observed for the 3-AP[±] fluorescence spectrum in aqueous solution.

A potential energy diagram for the ground- and excited-states of 3-AP[±] in DMSO (no proton transfer and proton transfer) and water is shown in Figure IV-3. Both the ground- and excited-states of 3-AP[±] are stabilized in water relative to DMSO. The difference between the equilibrium solute-solvent distances for the excited-state, r_e^E , and the ground-state, r_e^G , is greater for water than DMSO (no proton transfer) because of the increased H-bonding on the carboxylate groups in the excited-state in water. This greater displacement of the potential curves for water results in a higher energy Franck-Condon state and the blue shift observed in the aqueous absorption spectrum. The diagram also shows that after excitation and relaxation to the equilibrium excited-states, the fluorescence of 3-AP[±] in water will be red-shifted relative to the fluorescence in DMSO (no proton transfer).

The changes observed in the fluorescence quantum yield, ϕ_f , of 3-AP[±] as a function of the DMSO-water mixed solvent composition (Table III-10 and Figure III-19) also tend to support the above interpretation of the 3-AP[±] fluorescence spectra in the mixed solvents. Since the measured ϕ_f is the overall quantum yield for all the fluorescing species in solution, the decrease in the ϕ_f of 3-AP[±] in mixed solvents 5-7 can be explained on the basis of the formation of a species which has a ϕ_f smaller than the ϕ_f of 3-AP[±] in both water ($\phi_f \approx 25\%$) and DMSO ($\phi_f \approx 11\%$). As can be seen in Figure III-18, this decrease in ϕ_f correlates with the formation of the band at 410 nm. When the water concentration in the DMSO is sufficient to prevent

Figure IV-3

Diagram of the Potential Energy Curves for the
Ground- and Excited States of 3-AP[±] in DMSO (No
Proton Transfer and Proton Transfer) and in Water

Key

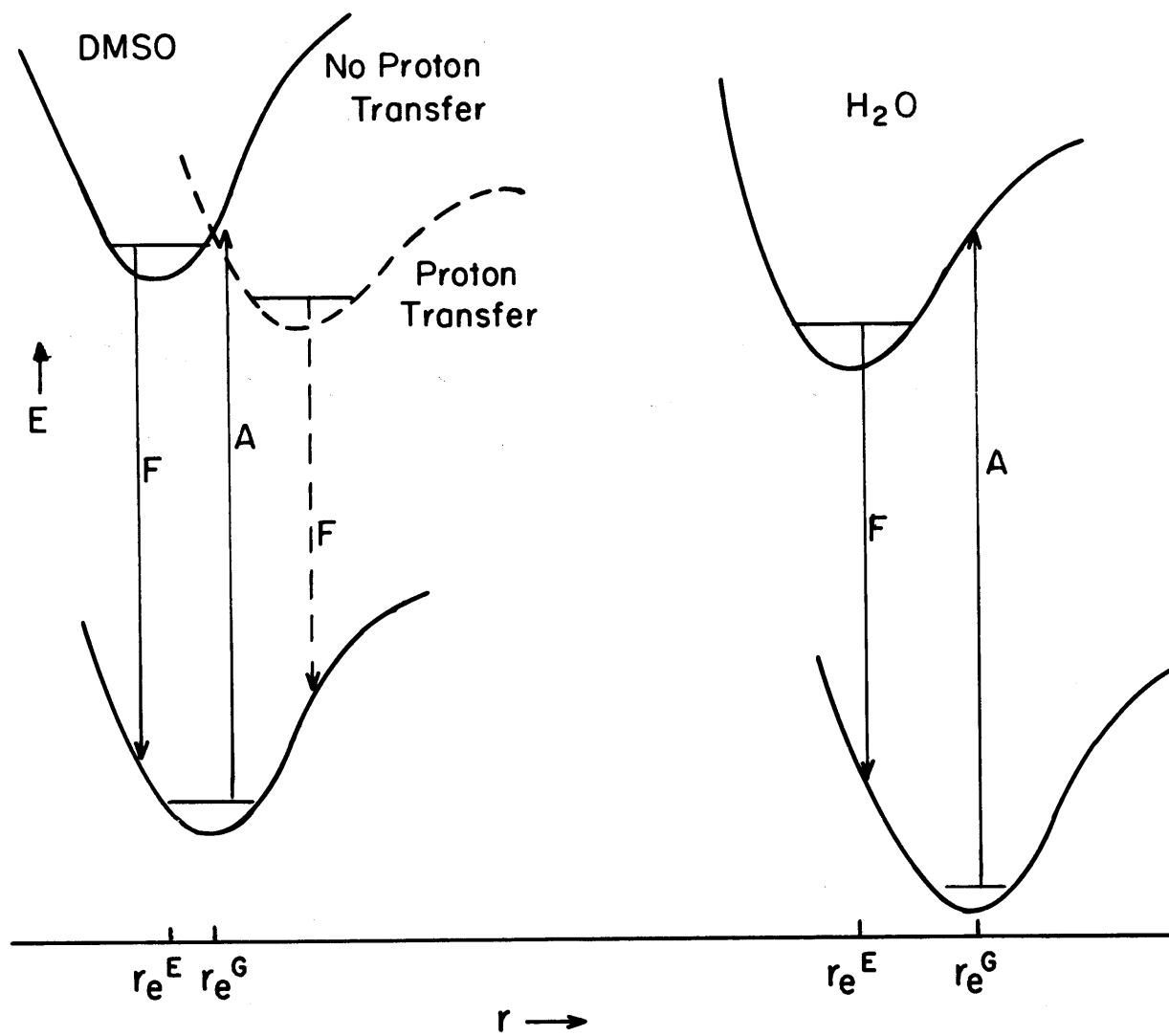
r_e^G = solute-solvent internuclear distance
in ground-state

r_e^E = solute-solvent internuclear distance
in excited-state

A = Absorption

F = Fluorescence

E = Energy



complete proton transfer in the excited-state, the 410 nm emission band develops and the ϕ_f decreases. This would indicate that the species responsible for the 410 nm band, i.e. the $3\text{-AP}^{\cdot-}$ in the DMSO environment but without proton transfer, is responsible for the decreased ϕ_f and that the ϕ_f for this species is less than 11%. Higher concentrations of water result in the formation of some of the water solvated $3\text{-AP}^{\cdot-}$ and ϕ_f increases.

2. Comparison of $3\text{-AP}^{\cdot-}$ Fluorescence and Luminol CL Spectra

The fluorescence spectrum of $3\text{-AP}^{\cdot-}$ in DMSO is very similar to the luminol CL spectrum in DMSO and the reaction product fluorescence spectrum in DMSO. These spectra are compared in Figure IV-4. The low intensity short wavelength band which is sometimes observed in the reaction product fluorescence spectrum in DMSO is also shown. As mentioned in Section III-A-3, this band is probably the result of either a secondary reaction product (formation of m-aminobenzoate) or solvent decomposition. These results are in agreement with the conclusions of White and Bursey (15) that the $3\text{-AP}^{\cdot-}$ is the emitting species and one of the products of the chemiluminescent reaction. The fluorescence spectra of $3\text{-AP}^{\cdot-}$ in mixed solvents 1-3 are also very similar to the $3\text{-AP}^{\cdot-}$ fluorescence spectrum in DMSO and the CL and reaction product spectra in the corresponding mixed solvents.

The luminol CL and $3\text{-AP}^{\cdot-}$ fluorescence spectra for mixed solvents 5-7 are compared in Figure IV-5. For each mixed solvent there appears to be a significant difference in the intensity of the

Figure IV-4

Comparison of the 3-AP⁺ Fluorescence, Luminol
CL, and Reaction Product Fluorescence Spectra
in DMSO

Key

—————	3-AP ⁺ Fluorescence
- - - - -	Luminol CL
- - - - -	Reaction Product Fluorescence

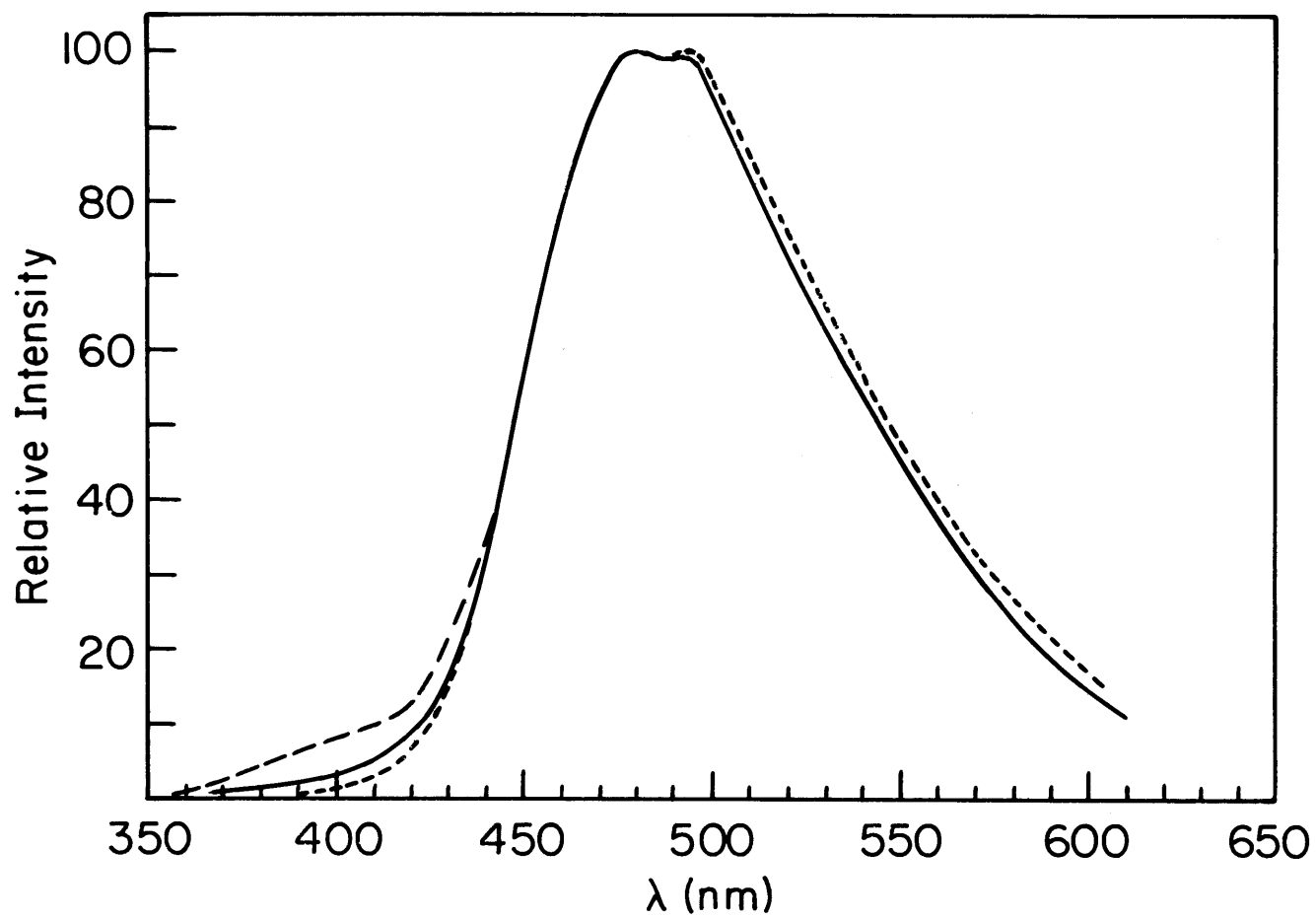
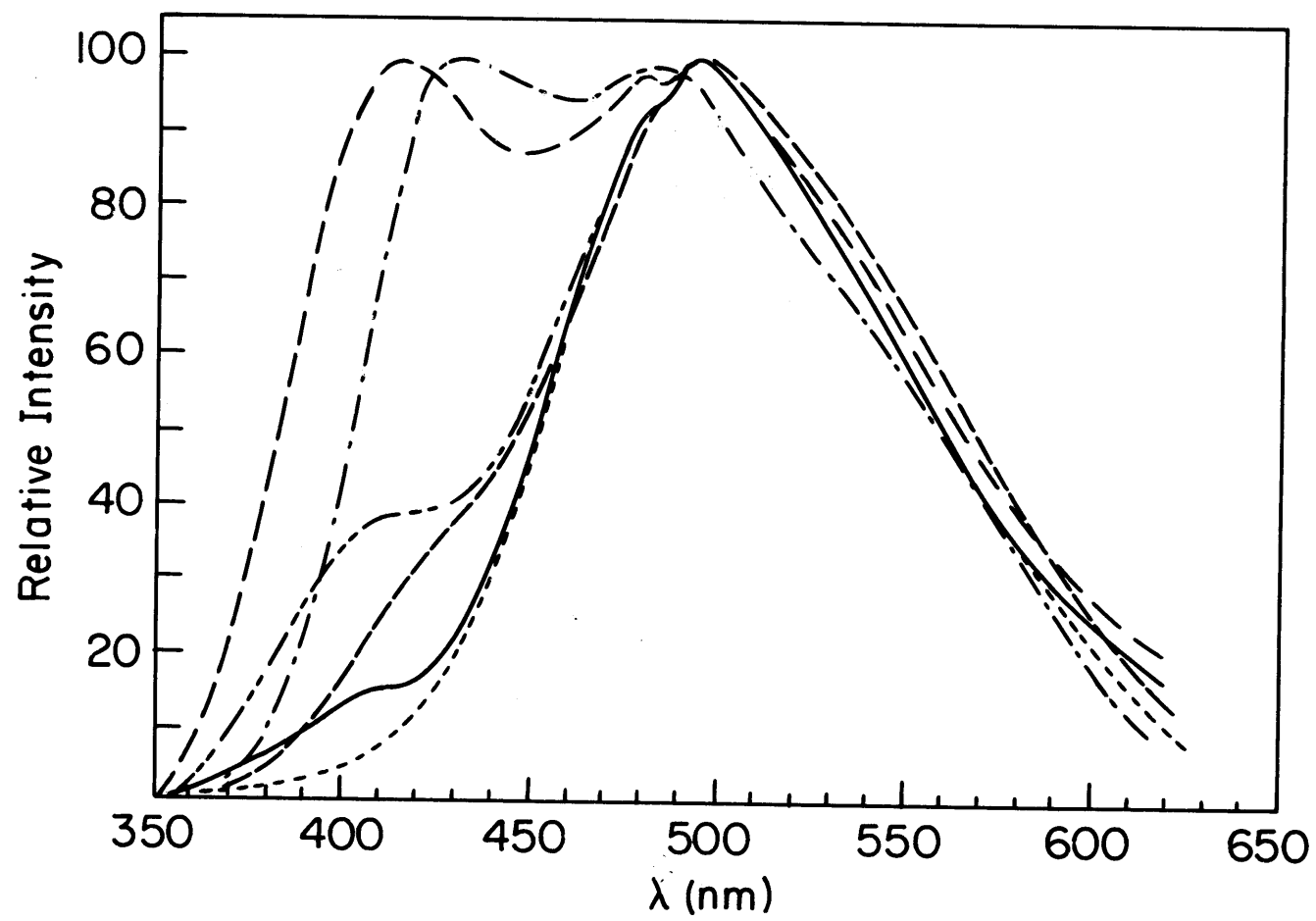


Figure IV-5

Comparison of the 3-AP⁺ Fluorescence and Luminol
CL Spectra in Mixed Solvents 5, 6, and 7

	<u>Key</u>	
Mixed Solvent 5:	-----	CL
	_____	Fluorescence
Mixed Solvent 6:	-----	CL
	-----	Fluorescence
Mixed Solvent 7:	-----	CL
	-----	Fluorescence



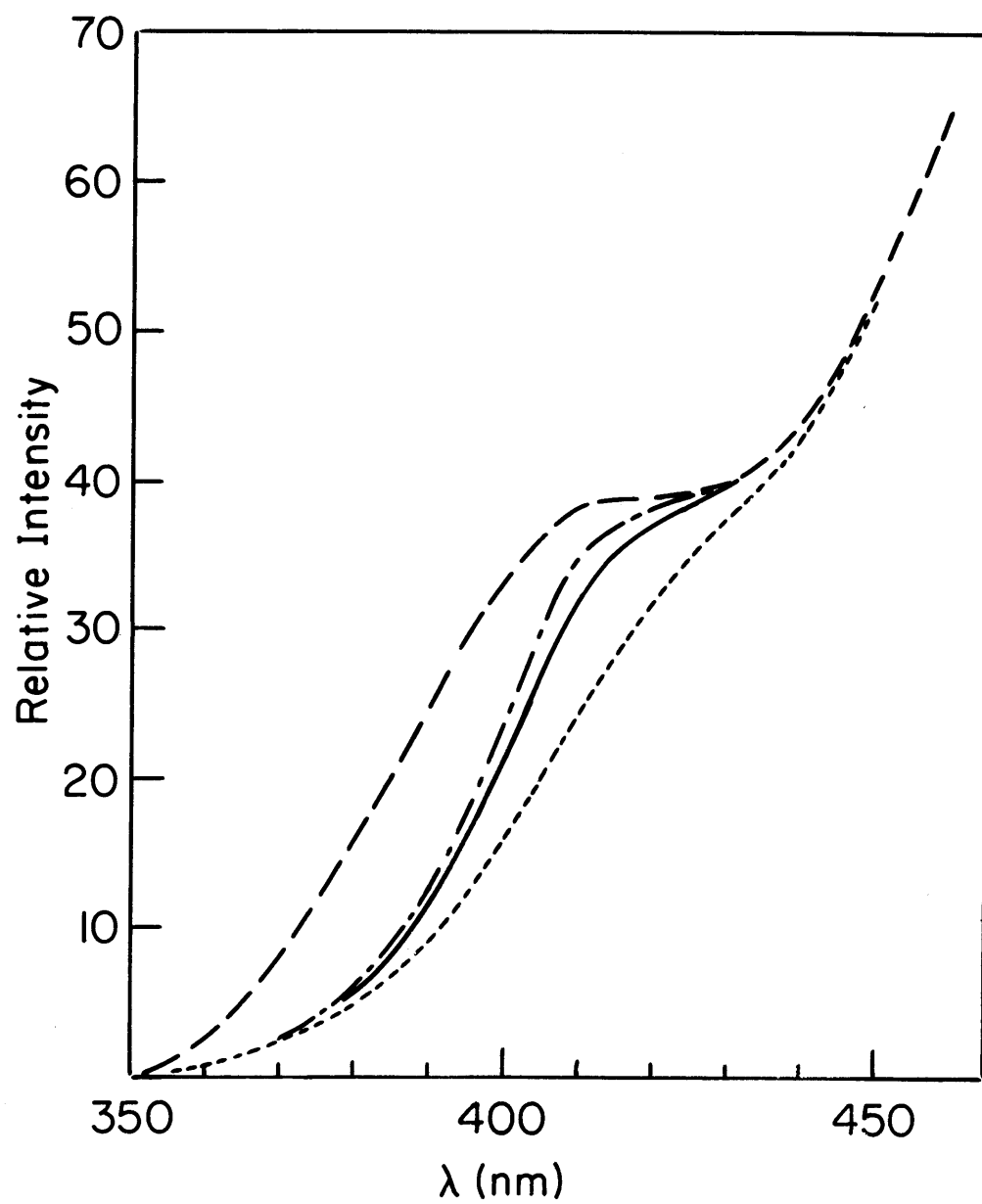
short wavelength bands of the 3-AP⁼ fluorescence and CL spectra. This band is consistently lower in the CL spectra. However, for a meaningful comparison of these spectra it is important to consider the conditions under which they were recorded. The 3-AP⁼ fluorescence spectra were obtained using a 25A emission slit. The rate of the chemiluminescent reaction and, thus, the light intensity fall off rapidly with increasing amounts of water. Therefore, the CL spectra in mixed solvents 5-7 required the use of larger slit widths (100A and 250A; see Section III-A-2). Since the reaction is slow in these mixed solvents, very little of the luminol is actually consumed while the solution is in the flow cell. As can be seen in Figure III-4, NaLUM in basic mixed solvents 5 and 6 shows absorption in the region where the intensity of the short wavelength band in the CL spectrum has been reduced. Figure IV-6 compares the short wavelength bands of the 3-AP⁼ fluorescence spectrum and the CL spectrum in mixed solvent 6 with the 3-AP⁼ fluorescence spectrum corrected for NaLUM absorption in basic mixed solvents 5 and 6 in the region from 370 nm to 430 nm. The fluorescence spectrum was corrected for mixed solvent 5 because the base concentration used to obtain the CL spectrum in mixed solvent 6 was higher than that used to obtain the NaLUM absorption spectrum in basic mixed solvent 6 (Figure III-4). Since a higher base concentration would tend to shift the NaLUM absorption spectrum in mixed solvent 6 to longer wavelengths because of the equilibrium between the monoanion and the dianion, it was felt that the absorption spectrum of NaLUM in basic mixed solvent 5 shown in Figure III-4 was a better

Figure IV-6

Correction of the 3-AP⁼ Fluorescence Spectrum
in Mixed Solvent 6 for Absorption by NaLUM in
Basic Mixed Solvents 5 and 6

Key

— — — — —	3-AP ⁼ Fluorescence in Mixed Solvent 6
- - - - -	Luminol CL in Mixed Solvent 6
— — — — —	3-AP ⁼ Fluorescence Corrected for NaLUM Absorption in Basic Mixed Solvent 6
—————	3-AP ⁼ Fluorescence Corrected for NaLUM Absorption in Basic Mixed Solvent 5



approximation of the absorption spectrum in mixed solvent 6 at the higher base concentration, particularly in the region where the 3-AP[≡] fluorescence spectrum was corrected.

The intensity of the 3-AP[≡] fluorescence spectrum in mixed solvent 6 was corrected by calculating the fraction of light which would be absorbed by the NaLUM solution in the region from 370 nm to 430 nm. For this calculation the CL cell length was assumed to be 0.5 cm. The extinction coefficients measured for NaLUM in mixed solvent 5 and 6 containing $2.5 \times 10^{-3} \text{N}$ KOH were used to calculate the absorbance of the NaLUM solution which was used to record the CL spectrum in mixed solvent 6. From Beer's law the absorbance at each wavelength can be used to calculate the fraction of light absorbed.

As can be seen in Figure IV-6, correction of the 3-AP[≡] fluorescence spectrum for NaLUM absorption using either the NaLUM absorption in basic mixed solvents 5 or 6 results in a good approximation of the CL spectrum observed in mixed solvent 6. The larger slit widths and/or slight differences in solvent composition could account for the remaining discrepancies. Although the CL spectra in mixed solvents 5 and 7 are also distorted in the short wavelength region by NaLUM absorption, it can be seen in Figure IV-5 that each of these CL spectra is very similar to the 3-AP[≡] fluorescence spectrum in the corresponding mixed solvent.

White and Bursey (15) have reported that in DMSO-water mixed solvents the CL peak at 425 nm develops at a slower rate than the 3-AP[≡] fluorescence peak at 425 nm. They suggest that this effect

may be the result of emission of the 3-AP[•] from slightly different solvent cages in the two excitation pathways. The spectra observed by White and Bursey (15) for the 3-AP[•] fluorescence and luminol CL in mixed solvents containing 17 mole % water-83 mole % DMSO and 30 mole % water-70 mole % DMSO are shown in Figure IV-7A and Figure IV-8A, respectively, along with the 3-AP[•] fluorescence and luminol CL spectra in mixed solvents 5 (Figure IV-7B) and 6 (Figure IV-8B) obtained in the present study. The compositions of the mixed solvents used by White and Bursey are very similar to the compositions of mixed solvents 5 and 6 (see Table III-3).

It is apparent from a comparison of the spectra in Figure IV-7 and Figure IV-8 that the fluorescence and CL spectra reported by White and Bursey not only differ from each other in each mixed solvent, they are also very different from the spectra observed in this investigation for similar mixed solvents. The only similarity is the CL spectrum in Figure IV-7A and the spectra in Figure IV-7B. It is suspected that in the study by White and Bursey the mixed solvent composition used to obtain the 3-AP[•] fluorescence spectrum was not the same as that used to obtain the luminol CL spectrum. Furthermore, the significant differences between their spectra and the spectra observed in this study suggest that the compositions reported for their mixed solvents are wrong. From the nature of their spectra, it appears that the mixed solvents contained more water than indicated.

The results of the present investigation clearly have

Figure IV-7

Comparison of the 3-AP[≡] Fluorescence and Luminol
CL Spectra Reported by White and Bursey (15) and
the 3-AP[≡] Fluorescence and Luminol CL Spectra Obtained
in the Present Investigation Using Mixed Solvent 5

Key

A: White and Bursey-

— — — — — 3-AP[≡] Fluorescence
————— Luminol CL

B: Present Investigation-

— — — — — 3-AP[≡] Fluorescence
————— Luminol CL

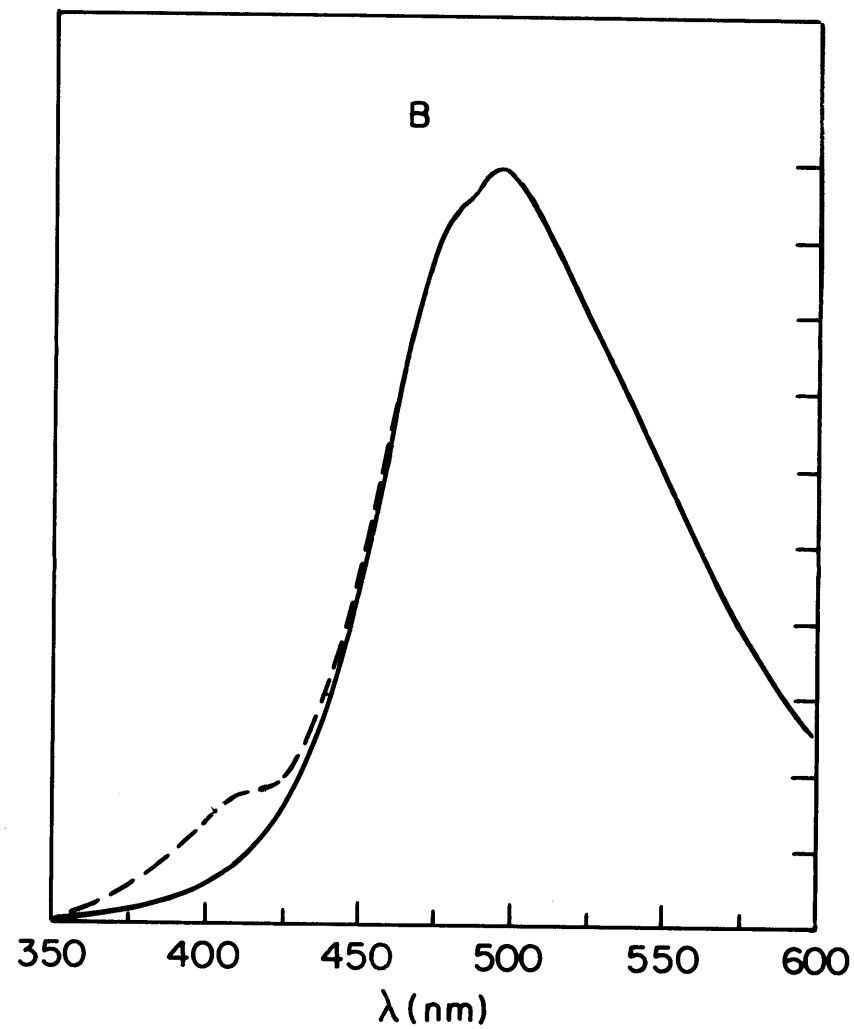
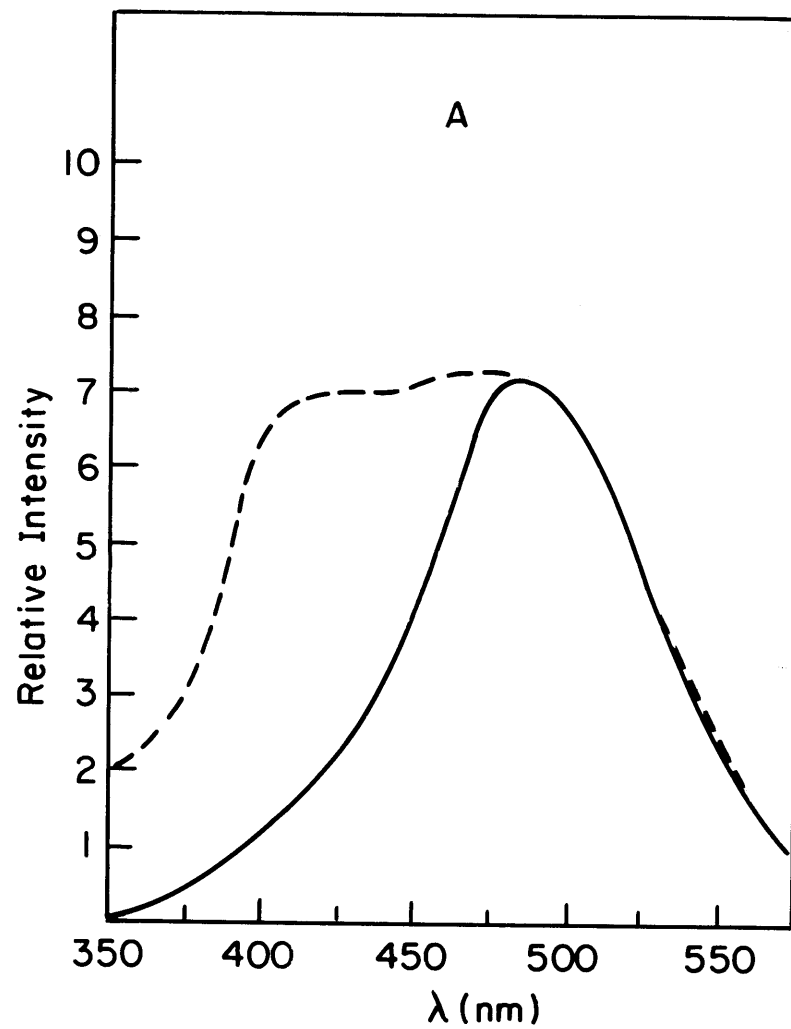


Figure IV-8

Comparison of the 3-AP⁺ Fluorescence and Luminol
CL Spectra Reported by White and Bursey (15) and
the 3-AP⁺ Fluorescence and Luminol CL Spectra Obtained
in the Present Investigation Using Mixed Solvent 6

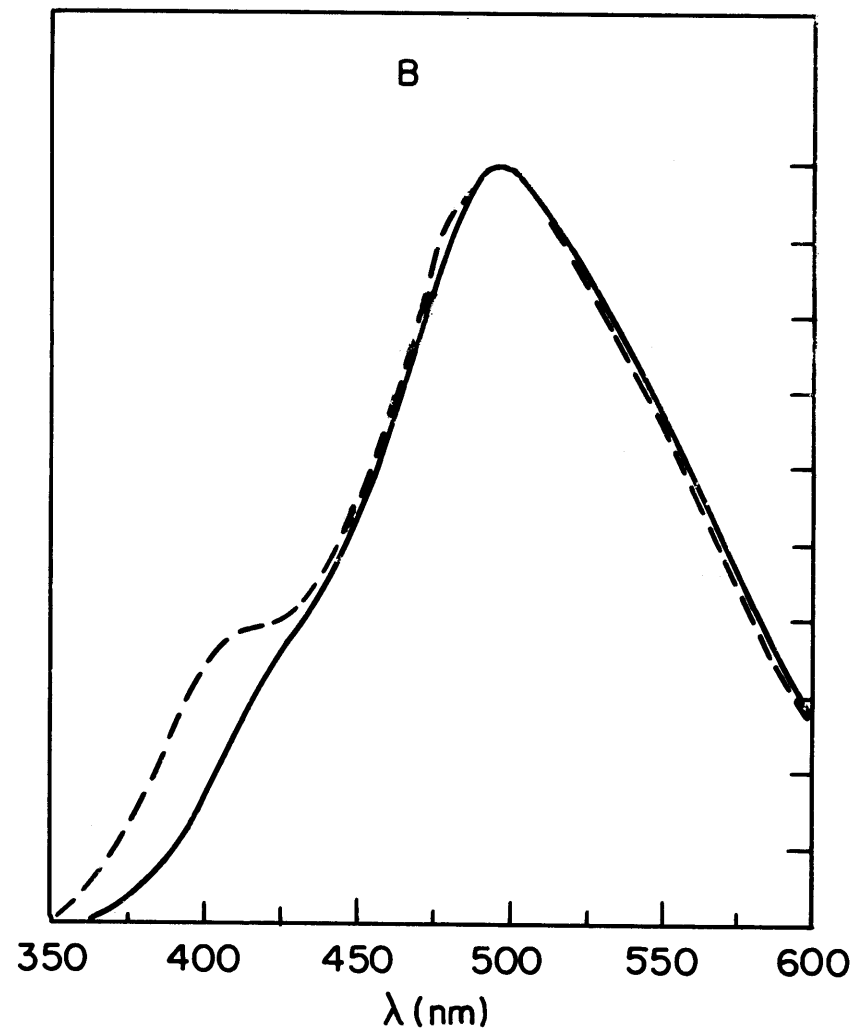
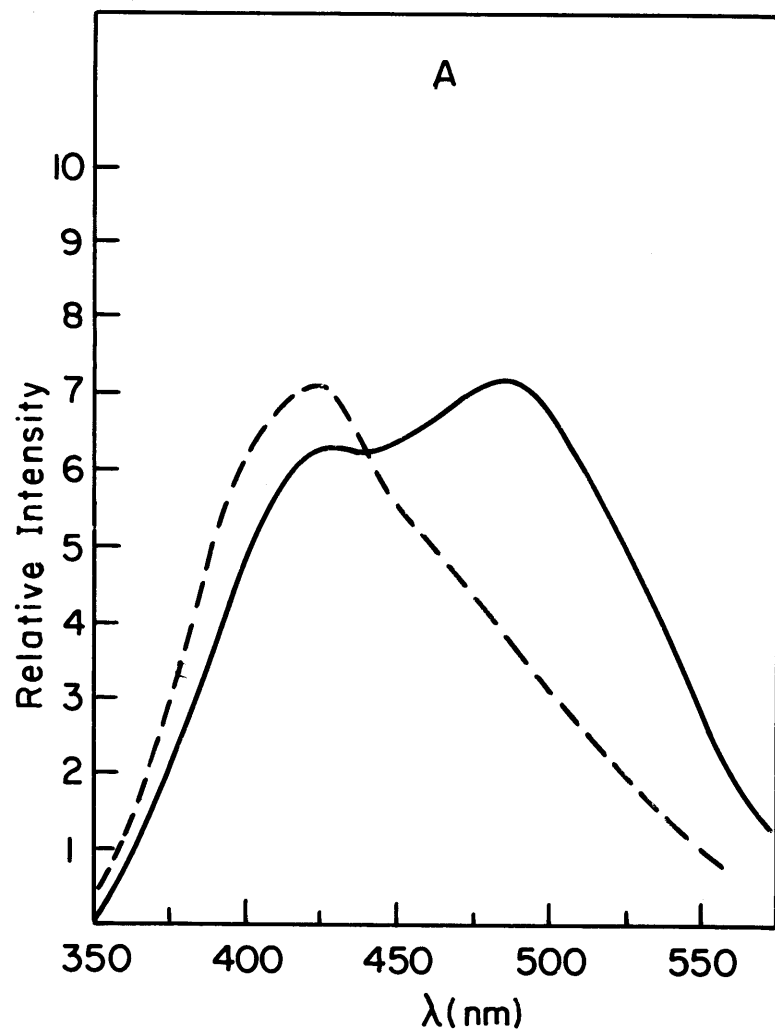
Key

A: White and Bursey-

— — — — — 3-AP⁺ Fluorescence
————— Luminol CL

B: Present Investigation-

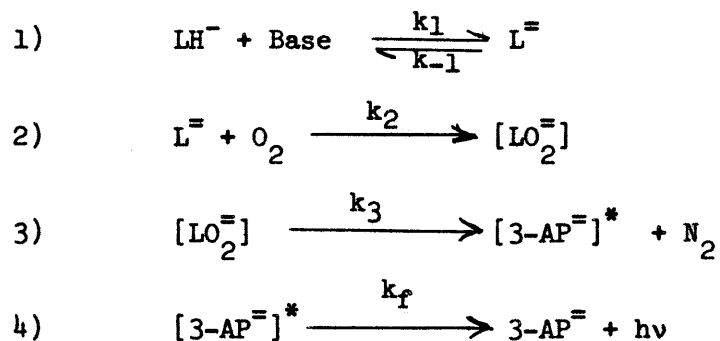
— — — — — 3-AP⁺ Fluorescence
————— Luminol CL



shown that there are no significant differences between the 3-AP⁻ fluorescence spectrum and the luminol CL spectrum in a given mixed solvent. The differences in the spectra reported by White and Bursey are not real. Since the 3-AP⁻ fluorescence spectrum and the luminol CL spectrum are very similar in each of these mixed solvents, both electronic and chemical excitation must produce the same solvent cage for the 3-AP⁻ excited-state.

C. Mechanism of the Chemiluminescent Reaction and Stopped-Flow Kinetics

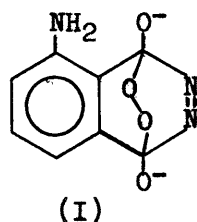
Although the kinetics of the luminol CL have been studied rather extensively in aqueous solution (12) (13), the only kinetic study in DMSO is that reported by White et al. (14). These workers proposed that the overall reaction in DMSO involved an equilibrium of the monoanion and dianion, followed by a slow oxidation step. This mechanism is shown below.



Using a low concentration of luminol ($4.0 \times 10^{-4}\text{M}$) and excess base ($6.0 \times 10^{-2}\text{M}$) and oxygen ($1.6 \times 10^{-3}\text{M}$), White found that the reaction was pseudo-first order, $k' = 2.5 \times 10^{-3}\text{sec}^{-1}$. Also, independently

varying the concentration of luminol, oxygen, and base showed that the reaction was first order in each of these reactants.

Intermediates have not been detected in the oxidation step in DMSO. The cyclic peroxide (I) (50) (51) (52) has been proposed as the intermediate, $[LO_2^=]$.



This type of intermediate is particularly attractive for visualizing the formation of nitrogen and $[3-AP^=]^*$ in reaction 3. White et al. (14) using O^{18} enriched oxygen gas have shown that the oxygen atoms introduced into the $3-AP^=$ framework came from the oxygen gas. The cyclic peroxide (I) is also attractive from an energetic standpoint. The elimination of nitrogen and cleavage of the peroxy bridge produce enough energy to excite the resulting $3-AP^=$ to its first excited-state.

The results of the present investigation agree with the overall mechanism proposed by White et al. (14). The correlation of the CL intensity and the dianion concentration has established that the dianion is the reactive intermediate in the chemiluminescent reaction in DMSO. However, contrary to the observations of White et al. (see Section IV-A-1), the spectral study of NaLUM in DMSO using BTO as the base has shown that the dianion can be formed

quantitatively in DMSO. The shapes of the CL decay curves obtained in the stopped-flow experiments are also consistent with the above mechanism. The presence of the maxima in the decay curves shows that the reaction scheme responsible for the luminol CL is a sequence of consecutive reactions of first and/or second order.

Since the proposed mechanism is a sequence of consecutive reactions of first and second order, there is no exact solution and a detailed kinetic treatment is impossible (53). However, independently varying the concentrations of reactants in the stopped-flow experiments resulted in some interesting changes in the CL decay curves, and some qualitative observations can be made.

The decay of $[3-AP^{\cdot-}]^*$ (reaction 4) is very fast, and the rate of the CL decay will be determined by the rates of reactions 1, 2, and 3. For very low concentrations of luminol and excess base and oxygen, reaction 1 is probably very fast and the pseudo-first order decay observed ($k' = 1.4 \times 10^{-1} \text{ sec}^{-1}$) is characteristic of either reaction 2 or 3, depending on their relative rate constants. Since the kinetic data of White et al. (14) were obtained using NaOH, the large discrepancy between the k' observed here and the k' reported by White et al. ($2.5 \times 10^{-3} \text{ sec}^{-1}$) is undoubtedly due to the fact that they were using DMSO which contained a significant amount of water (NaOH is virtually insoluble in dry DMSO). The stopped-flow experiments using the DMSO-water mixed solvents have clearly shown that relatively small amounts of water in the DMSO greatly decrease the rate of the CL decay.

The CL decay curves obtained for the low concentrations of

BTO (excess luminol and oxygen) and the low concentrations of oxygen (excess luminol and BTO) are quite interesting. Although the maxima of these decay curves do not completely disappear, the curves do become pseudo-first order after the maxima. These observations suggest that at these concentrations the final step in the reaction scheme (reaction 3) is controlling the decay after the maximum. It should also be noted that the k' 's for these concentrations ($k' = 6.8 \times 10^{-2} \text{sec}^{-1}$ for low BTO and $k' = 1.5 \times 10^{-1} \text{sec}^{-1}$ for low oxygen) are very similar to the k' for the low concentration of luminol ($k' = 1.4 \times 10^{-1} \text{sec}^{-1}$). Since these k' 's are of the same order of magnitude, it appears that for these extreme concentrations there is one slow step in the reaction scheme which is controlling the CL decay, and this step is essentially independent of the reactant concentration which is varied. These results indicate that the decomposition of the $[\text{LO}_2^{\bullet}]$ is the rate controlling step. If this is the case, then the measured k' is a good estimate of the rate constant of the decay of $[\text{LO}_2^{\bullet}]$ since this is a unimolecular reaction.

The preliminary stopped-flow experiments in the present investigation have shown that in order to obtain reliable CL decay curves using DMSO, extreme care must be taken to ensure that the DMSO is dry and that the BTO-DMSO solutions are freshly prepared and completely deoxygenated. Interpretation of the decay curves presents an even greater problem. As mentioned above, the reaction scheme for the luminol CL is complex. Therefore, it is believed that a more detailed and meaningful interpretation of the stopped-flow CL

decay curves will require the use of a computerized kinetics program, such as the one developed by Lytle (54).

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